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Nancy Holbrook April 9, 2017

Fungal Endophytes in *Asclepias*

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2017

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Interest in the ecological importance and presence of fungal endophytes, generally non-pathogenic fungi that live inside plant tissues, has increased among scientists in recent years. Studies of endophytes in plants from the equator to the arctic, and from tropical trees to agricultural grasses, demonstrate the remarkably widespread nature of fungal endophyte infection. This thesis project focuses on the cultivation and identification of fungal foliar endophytes, fungal endophytes found growing within leaves, in four *Asclepias* (milkweed) species: *A. curassavica, A. incarnata, A. syriaca,* and *A. tuberosa.* Incidence and prevalence of endophytes, as well as composition of the fungal endophyte communities in the four species are compared and discussed. Results indicate a diverse community of fungal foliar endophytes within the leaves for *Asclepias*. I discuss the implications for milkweed-herbivore interactions.

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INTRODUCTION

Fungal endophytes are fungi that reside within plant tissues without causing outward, visually-evident disease symptoms (*e.g.,* lesions or rot). Structures such as stems, leaves, petals, and bark are commonly colonized by these fungi (Faeth & Fagan, 2002). Fungal endophytes can grow both intercellularly and intracellularly within host plants, and are distinguished from fungal mycorrhizae by the absence of external sheath structures (mantles), which are often found in mycorrhizae (Saikkonen *et al.* 1998). Fungal endophytes are pervasive throughout plant species (Arnold & Lutzoni, 2007). Indeed, plants examined from an enormous variety of habitats, both terrestrial and aquatic, have been shown to harbor endophytic fungi (Stone *et al.* 2000). The ubiquity of fungal endophytes across various habitats and plant genera raises questions regarding the ecological roles that endophytic fungi may play, and studies have shown that endophyte infection can elicit a wide range of plant responses. Endophytic fungi, for example, have been shown to produce alkaloid compounds that can increase host plant resistance to herbivores (Faeth & Fagan, 2002), and foliar endophytes of *Theobroma cacao* offer seedlings protection against a virulent foliar pathogen (Arnold *et al.* 2003). Endophytes, however, are not always beneficial to their plant hosts. Maize and banana plants, for example, have decreased photosynthetic efficiency as a result of infection with the endophytes *Colletotrichum musae* and *Fusarium moniliforme* (Pinto *et al.* 2000). In addition, fungal endophytes can increase water loss during drought conditions in some plants (Arnold & Engelbrecht, 2007).

Research on endophytic fungi has increased drastically in recent years as biologists have gained interest in the abundance and tremendous diversity of these fungi. However, only endophytes in agronomic grasses have been extensively studied. Very little is known regarding the types and functions of fungal endophytes that exist within the majority of plants found in

nature (Faeth & Fagan, 2002), and only a fraction of that work is on fungal foliar endophytes of herbaceous plants, the focus of this study.

Characterizing Endophytes

Fungal endophytes can be divided into two major classes: clavicipitaceous (C) endophytes and non-clavicipitaceous (NC) endophytes. Of these two groups, C-endophytes have been studied more extensively, as these fungi are found within grasses important to the agricultural industry. NC-endophytes inhabit non-vascular plants, ferns, conifers, and angiosperms (Rodriguez *et al.* 2009). Here, I will focus on NC-endophytes. As mentioned previously, fungal endophytes may be found in nearly all plant structures, including leaves, stem, roots, petals, and bark (Faeth & Fagan, 2002, Saikkonen et al. 1998). This project narrows its focus to the foliar fungal endophytes of *Asclepias*, which I will often refer to as "fungal endophytes" or "endophytes" for simplicity.

Prevalence of fungal endophytes

Fungal endophytes have been found in all plant species studied to date, but are especially bountiful in mature woody plants, which can contain hundreds of differing fungal species (Faeth & Fagan, 2002). Previous studies have shown endophytes to be associated with a wide variety of plant types, including but not limited to Mediterranean oak trees (Morrica & Ragazzi, 2007), the tropical *Theobroma cacao* chocolate tree (Arnold & Engelbrecht 2007), rye and fescue grasses (Vinton *et al.* 2001), and mangrove trees from southern India (Suryanarayanan *et al.* 1998). Endophytes are widely taxonomically and spatially distributed. A survey of eight localities of varying latitude from the equator to the arctic, for example, isolated 8456 different strains of endophytic fungi, and endophytes were found in every major plant lineage that was sampled. While incidence of endophyte infection was generally lower as latitude increased, fungal strains

could still be found in samples taken from an arctic tundra site in Nunavut, Canada (Arnold & Lutzoni 2007).

Why study fungal endophytes in milkweeds? Milkweed diversity and its influence of the fungal community

Here, I conduct the first investigation of fungal endophytes in milkweed plants (*Asclepias* spp.). *Asclepias* is a widely-distributed genus of herbaeceous plant that is host to a diverse community of specialist herbivores. These plants are well known because of the milky latex they produce, which can contain high levels of secondary metabolites that can be toxic to herbivores (Vickerman & Boer, 2002). Specifically, milkweeds contain a class of chemicals termed cardenolides (Zalucki *et al.* 2001). Cardenolides are steroid compounds that interfere with the activity of sodium/potassium ATPase, making them toxic to many animals (Agrawal *et al.* 2012). Cardenolide concentration differs greatly among the various species of milkweeds (Agrawal & Fishbein, 2008). Due to this variation in chemical makeup, it seems reasonable to expect that different milkweed species may harbor different communities of fungal endophytes. Indeed, it has been shown in other systems that leaf biochemistry influences the composition of fungal endophyte communities (Estrada *et al.* 2013, Kembel & Mueller, 2014).

Given the ecological importance of *Asclepias* spp. and the importance of fungal endophytes in shaping plant ecology, we were interested in determining whether milkweed plants contained cultivable endophytes that could alter their association with the abiotic and biotic environment. Given milkweed diversity in terms of distribution, ecology and chemistry, we were also interested in investigating whether different species of milkweed harbor differing endophytic communities. These questions were addressed via the sterilization of milkweed leaf

samples, the cultivation and sequencing of fungal endophytes, and *in vitro* assays with cultivated endophytes and plant secondary metabolites from several species of milkweed.

Why study fungal endophytes in milkweeds? The potential impact of milkweed fungal endophytes on monarch butterfly ecology

Furthermore, the examination of fungal endophytes in the *Asclepias* (milkweed) plant genus is of particular interest due to the role milkweed species play in monarch butterfly ecology. Milkweeds are the host plant for monarch butterflies (*Danaus plexippus*), which are well known for their North America migration and whose North American population is in decline, in part because of loss of these critical host plants (Pleasants & Oberhauser, 2013). Monarch butterflies (*Danaus plexippus*) utilize milkweed plants as a food source and site for oviposition (De Roode *et al*. 2008). In North America, monarchs can be found in multiple areas, such as the eastern United States and southern Florida. The location of a monarch population affects their utilization of milkweed species; for example, the tropical milkweed *A. curassavica* is more common in southern Florida than is the *A. incarnata* swamp species. Previous research has shown that monarch populations can utilize at least 27 different species of milkweed plants as a food source (De Roode *et al.* 2008).

Monarch populations are sometimes affected by infection with a particular widespread protozoan parasite, *Ophryocystis elektroscirrha*. *O. elektroscirrha* is propagated via spores that can contaminate egg or host milkweed leaf surfaces. Upon the ingestion of spores by monarch caterpillars, the parasite reproduces in the larval hypoderm and eventually produces spores that settle on the adult monarch's scales. Spores can then be shed by the monarch during oviposition, potentially infecting the next generation of monarch offspring (De Roode *et al.* 2008).

Previous research has demonstrated differences in *O. elektroscirrha* infection rates between monarch larvae that have been reared on different species of milkweed. In one study, monarchs reared on the milkweed *Asclepias curassavica* demonstrated lower parasite loads and rates of infection than monarchs reared on *Asclepias incarnata* (De Roode *et al.* 2008). As previously mentioned, these milkweed plant species are known to differ in cardenolide levels; cardenolide concentration in *A. curassavica* exceeds that of *A. incarnata* by 13 fold. However, the two species share a similar nutritional and morphological profile (Tao *et al.* 2014, De Roode *et al.* 2008). It is unclear whether the observed differing medicinal properties of the two species is directly due to cardenolide concentrations alone. Monarchs have been shown to harbor characteristic gut bacteria throughout their life stages (Kingsley, 1972); thus, it is possible that the cardenolide concentration among different milkweed species indirectly affects monarch disease resistance via some mechanism of altering gut bacteria, or possibly gut fungi. Such gut microbes are largely acquired from the environment as the animals eat their hosts plants.

To our knowledge, there are no published studies regarding the fungal endophytes that may be present among different milkweed species. If the communities of fungal endophytes found in the various species of milkweed do indeed differ, then these differing endophytic communities may play a role in moderating monarch disease resistance either directly or indirectly. In a direct effect scenario, we might expect that certain fungal endophyte species, once consumed by the monarchs, discourage parasite infection, while an indirect effect scenario may involve certain fungal endophytes altering gut bacteria composition in a manner which in turn affects disease resistance. However, to begin to understand how and if fungal endophytes may affect monarch disease resistance, we must first have a better grasp of the fungal endophytic communities present within milkweed plants, and how these are established and maintained

within the monarch gut. My research focuses on the latter through isolation and identification of fungal foliar endophytes from several common milkweed species. I compare both endophyte abundance and the composition of endophyte communities among these different milkweed host plant species.

OUTLINE

In beginning to examine the fungal endophyte communities in milkweed plants, my research first focuses on determining the prevalence and incidence of infections by cultivable fungal foliar endophytes in several *Asclepias* species. In part two of the study, by classifying the collected fungi into fungal morphotype groups, I begin to explore whether different plant species harbor different communities of fungal endophytes. Part three of the study focuses on determining whether cardenolides affect the growth of fungal endophytes via *in vitro* cardenolide inhibition assays. I then end the thesis with conclusions, focusing on future work in relation to milkweed foliar endophytes.

METHODS

PART I: Cultivation of Fungal Endophytes

Sample Collection

The following milkweed species were sampled from several sites between August and November of 2016: *A. curassavica*, *A. incarnata*, *A. tuberosa*, and *A. syriaca*. Samples taken from outdoor locations in Atlanta, Georgia (Fernbank Museum, Gerardo Garden, De Roode Garden) and Miami, Florida (Miami Yard A, Miami Yard B) were collected from fully developed plants via clipping cuttings 15-30 cm in size, except for *A. incarnata* samples from the Carter Center, which are described below. The cuttings included several mature leaves as well as a sampling of new growth. In addition, *A. incarnata* and *A. curassavica* samples from the Michael Street Greenhouse were collected by removing two to four individual leaves per plant sampled. Care was again taken to ensure that both mature leaves and young leaves were selected. *A. incarnata* plants sampled from the Carter Center were all young, standing 3-9 cm in height. Carter Center samples were thus collected as cuttings no more than 5-6 cm in size. As a result of the small size of Carter Center plants, leaves from these cuttings were pooled and grouped by plant species and plant number before sterilization and fungal endophyte cultivation. Samples collected from all other sites were grouped by plant species, plant number, and leaf number, allowing collection of data by leaf. In addition to the *Asclepias* spp. samples, samples were taken from plants of three other genera at the Gerardo Garden site (*Callicarpa americana* (American Beauty Berry), *Rosa* sp. (Rose) and *Vaccinium* sp. (Blueberry)).

Table 1: Sample Collection Locations. See Appendix A for map of all locations.

Sample Plant Species	Locations of Collection
Asclepias curassavica	Michael St. Greenhouse (Atlanta, GA)

	Gerardo Garden (Atlanta, GA) Miami Yard A (Miami, Florida) Miami Yard B (Miami, Florida)
Asclepias incarnata	Michael St. Greenhouse (Atlanta, GA) De Roode Garden (Atlanta, GA) The Carter Center grounds (Atlanta, GA)
Asclepias tuberosa	Fernbank Science Center (Atlanta, GA)
Asclepias syriaca	Fernbank Science Center (Atlanta, GA)
Callicarpa americana	Gerardo Garden (Atlanta, GA)
Rosa sp.	Gerardo Garden (Atlanta, GA)
Vaccinium sp.	Gerardo Garden (Atlanta, GA)

See Table 2 and Table 3 in Appendix B for detailed breakdown of samples collected by plant and by leaf.

Sterilization

A modified version of the leaf sterilization protocol found in Slack, Arnold, & Strobel, 2012 was used for this study. A 0.5% NaOCl solution was prepared using Clorox concentrated bleach (8.25% NaOCl) and ultrapurified water. Each leaf was prepared for sterilization via a 30 second wash in running tap water to remove any debris. Leaves were then gently dried with paper towels and cut with small dissection scissors into rectangles with dimensions of approximately 1mm * 2mm. Leaf pieces were taken primarily from the outer leaf edges. In a small Petri dish, the pieces of each leaf were placed in a primary wash with the prepared 0.5% NaOCl solution. After leaf pieces were immersed in the bleach solution, the Petri dish was closed and agitated by hand for a period of two minutes. The solution was promptly drained from the leaves, and a secondary wash of 70% ethanol was applied using a two-minute hand-agitation method as in the primary 0.5% NaOCl wash. The ethanol solution was subsequently drained and samples were transferred to a biosafety cabinet.

After transfer to the biosafety cabinet, Petri dish lids were removed to encourage evaporation of excess ethanol solution. Excess moisture was also removed via blotting with

autoclaved Kim wipes. When samples appeared dry, sterilized forceps were used to plate individual leaf pieces onto 100 mm Petri dishes with malt extract agar. Six to eight leaf pieces were plated equidistantly on each plate. Plates were then wrapped with parafilm to prevent moisture loss and stored at 25°C. In total, 2672 pieces were monitored for infection, and 2328 of these pieces were from *Asclepias* spp..

Monitoring Infection and Isolation of Fungal Strains

All plated samples were monitored every 2-3 days for endophyte emergence, and the presence or absence of fungal endophyte growth was recorded. Plates were monitored for at least 30 days. Any fungal growth was cut out of agar in a 2mm * 2mm plug using a flame sterilized scalpel. Each fungal plug was then transferred to a new malt extract agar plate for isolation.

Analysis of Infection Prevalence Data

Each leaf piece was scored for presence or absence of fungal endophyte growth. Prevalence data (the proportion of leaf pieces showing infection within a leaf) were analyzed using General Linear Mixed Models (binomial distribution) using the lme package in R (ver 3.3.1). Plant species, and in some cases location, were fixed effects, and leaves and plants were random effects. Analyses included: 1) comparison of fungal endophyte prevalence in samples collected from the greenhouse and outdoor settings across two plant species (*A. incarnata*, *A. curassavica*); 2) Comparison of fungal endophyte prevalence in samples collected from plants of four genera at the same location (*A. curassavica*, *Callicarpa americana, Rosa* sp., and *Vaccinium* sp.); 3) comparison of fungal endophyte prevalence in *A. incarnata* collected from two different locations; 4) comparison of fungal endophyte prevalence in *A. curassavica* collected from three different locations; and 5) comparison of fungal endophyte prevalence across all sampled *Asclepias* species (*A. tuberosa, A.incarnata, A. syriaca, and A. curassavica*). In addition,

differences in incidence (the proportion of leaves infected) were analyzed using Chi-square tests or Fisher's Exact tests.

PART II: Identification of Fungal Endophytes

Visual Fungal Endophyte Classification in Morphotypes

All fungal endophyte isolate plates were visually examined for classification into morphotype groups. Classification criteria included coloration, density of growth, and texture of fungus. Using these three criteria, the fungal endophyte isolates were classified into a total of 30 morphotype categories, which are described in Table 4.

Analysis of Morphotype Data

To begin to assess whether differences in fungal endophyte composition among different *Asclepias* species were statistically significantly different, we considered only data from *Asclepias* leaf pieces that produced a fungal endophyte that was classified into one of the morphotype groups. Fungal endophyte community composition across plant species and locations was visually inspected through construction of stacked bar plots in ggplot (R ver 3.3.1) to illustrate morphotype proportions within each group. One *A. syriaca* leaf piece with a unique morphotype (not found in any other sample) was removed before further analysis.

Using the Vegan package of R (ver 3.3.1), sampling effort and morphotype richness across *Asclepias*. spp were determined, and a clustering dendrogram and a Non-metric Multidimensional Scaling (NMDS) plot were constructed based on Morista-Horn dissimilarity indices, with each leaf representing a sample. Permutational multivariate analysis of variance tests were performed, based on the Morisita-Horn dissimilarities, using the adonis function from the vegan package to compare fungal endophyte community composition across *Asclepias* spp., with leaves nested within plants.

Molecular Identification of Fungal Endophytes

DNA Extraction

To begin to assess the robustness of the visual morphotype classifications and to assign each morphotype to genus, a portion of the Inner Transcribed Spacer (ITS) region of DNA was amplified and sequenced from a subset of samples. A DNA extraction protocol from the Vilgalys lab was modified and used for this study. DNA samples were collected from freshly plated (~1 week old) fungal endophytes. A heat sterilized scalpel was used to scrape the surface of the agar, lifting fungal material. Enough fungus from each sample was collected in order to completely cover the bottom of a 1.5 mL Eppendorf tube. 250 μL of 2X CTAB extraction buffer was added to each sample, and a sterilized pestle was used to crush mycelia until the solution appeared homogenous. Another 250 μL of 2X CTAB extraction buffer was then added to each sample; samples were mixed via vortex for 15-20 seconds.

After vortexing, samples were placed in a heating block set to 65°C for 30 minutes. 500μL of chloroform-isoamyl alcohol was added to each sample, and samples were shaken vigorously to emulsify the extraction solution. Each sample was then placed into a centrifuge set to 13,000 rpm for 12 minutes. The upper aqueous supernatant of each sample was removed and placed into clean Eppendorf tubes, each containing 300 μL of isopropanol at -20°C. Samples were placed in a -20°C freezer overnight. The next day, the DNA precipitate was collected by again placing the samples in a centrifuge set at 13,000 rpm for seven minutes. The supernatant was poured off, and 1 mL of -20°C 70% ethanol was added to each sample. The samples were again centrifuged at 13,000 rpm for two minutes. The solution was poured from each sample tube, and tubes containing DNA pellets were inverted on a sterile Kim wipe and left to dry for

approximately three hours. DNA pellets were each resuspended in 50 μL of sterile, molecular grade water and stored in a -20°C freezer.

PCR, Sequencing and Identification

DNA amplification was performed using primer ITS 4 (5' TCCTCCGCTTATTGATATGC) and ITS 5 (5' GGAAGTAAAAGTCGTAACAAGG). The PCR reaction was from White *et al*. 1990. The following PCR program was used: 1) a beginning period of five minutes at 95°C was completed for strand separation; 2) 30 cycles of 45 seconds at 95°C, 45 seconds at 50°C, 90 seconds at 72°C; and 3) hold at 72°C for 10 minutes. Samples were purified using the Qiagen PCR purification kit before being sent for sequencing.

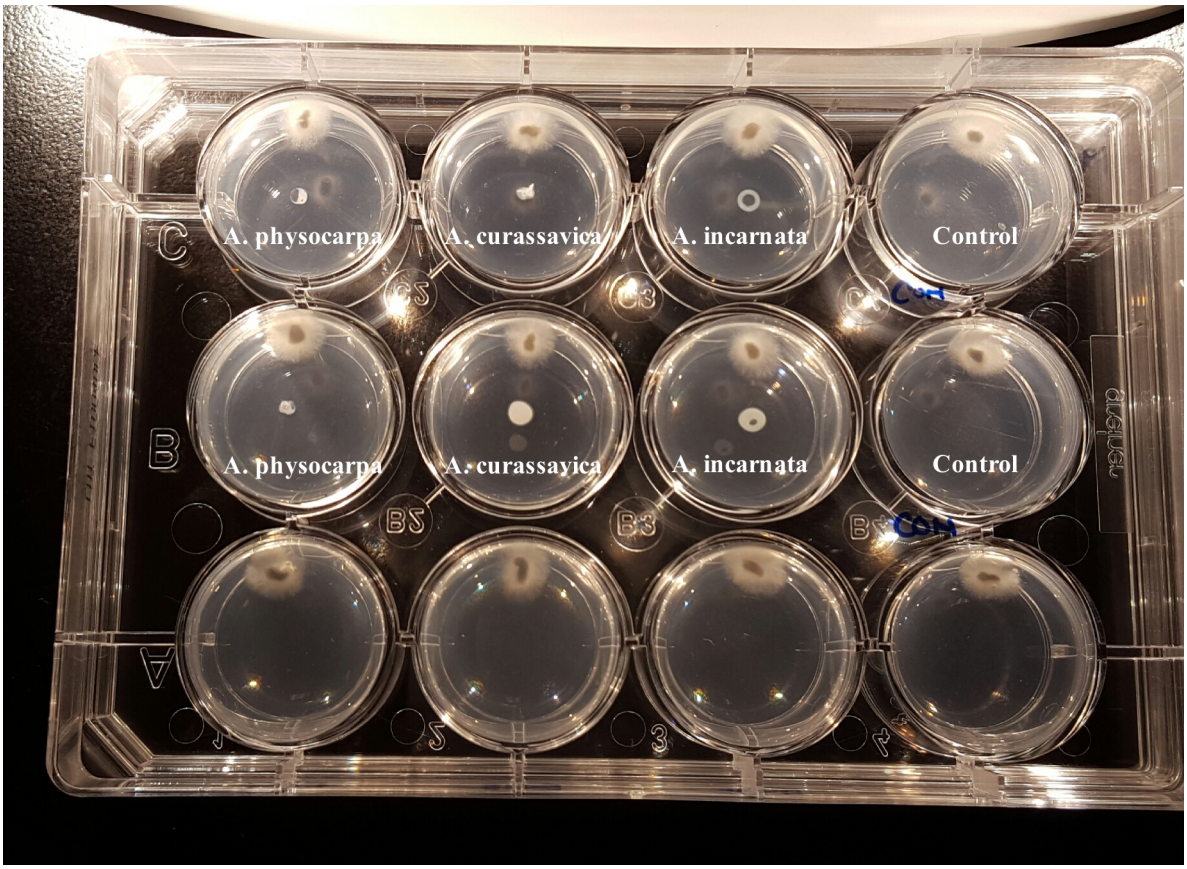
Trimmed sequences were used to BLAST against the NR database at NCBI.

Assays of Fungal Growth Inhibition by Milkweed Latex

Zone of inhibition assays were performed to determine whether the differing cardenolide contents among the four species of *Asclepias* played a role in discouraging fungal endophyte growth. A modified version of the well plate assay described in Kerr, 1999 was utilized.

Cell culture plates containing twelve 22 mm wells each were used for the assays; the bottom of each well was covered with malt extract agar to allow for cultivation of the fungi. Each of the wells was inoculated with a very small amount (ball of ~1-2 mm diameter) of fungal material from a fresh culture of the corresponding fungal endophyte. The inoculate was placed about 3 mm from the edge of each well. Plates were then stored for 24 hours to allow mycelia to begin to grow.

Sets of assays were performed in duplicate for each fungal endophyte tested with one set as follows: one control well with no latex, one well with *A. incarnata* latex, one well with *A. curassavica* latex, and one well containing *A. physocarpa* latex (see Ex. 1). Milkweed latex donor species were chosen based on their cardenolide content: *A. incarnata* represents a low concentration of cardenolides, while *A. curassavica* has a moderate to high concentration of cardenolides and *A. physocarpa* has a high cardenolide concentration (Agrawal et. al 2008, Sternberg et al. 2012). After the 24 hour primary period of mycelia growth, 1 µl of milkweed



Example 1: Cardenolide zone of inhibition assay setup. A plate with twelve wells was used for each fungal endophyte tested. For each endophyte, the assay was performed in duplicate with latex utilized from *A. incarnata, A. curassavica, and A. physocarpa.*

latex was collected from each of the previously mentioned species for each of the wells with the exception of the control wells. Note that *A. physocarpa* host plants had smaller volumes of latex

and as a result, some *A. physocarpa* test wells may have had <1 µl of latex applied. The collection involved stripping leaves off of the plant and draining emergent latex with a pipette. Each latex droplet was added to the center of the corresponding well. Plates were wrapped in parafilm to prevent moisture loss, stored at 25°C, and observed and photographed each day for a period of five days.

Data were collected by performing measurements on plate photographs each day for four days. The software Image J was used to measure the distance from the center of fungal endophyte growth to the edge of growth in the direction of the central latex droplet. For a given row, distances measured in each well were divided by the distance measured in the control well from the same row to generate a measure of relative growth.

Results

Fungal endophytic infection prevalence and incidence in *Asclepias* plants grown in greenhouse and natural settings

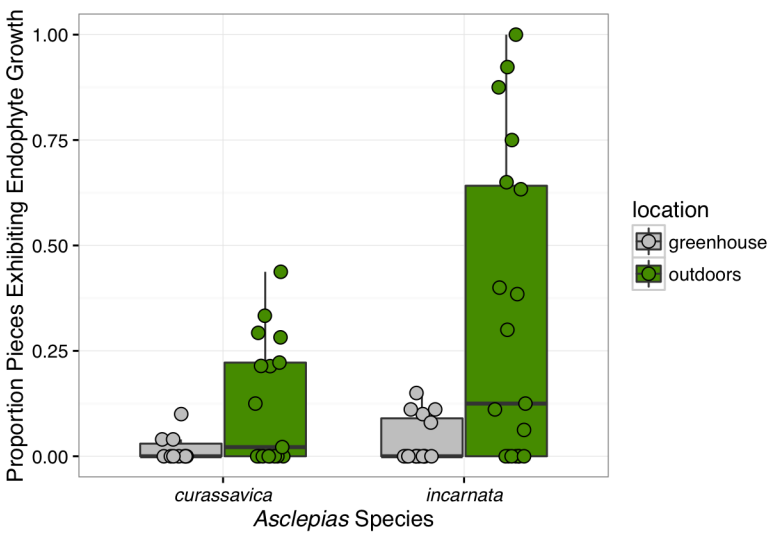


Figure 1: Proportion of leaf pieces exhibiting fungal endophytic infection within *A. curassavica* and *A. incarnata* grown in greenhouse and natural outdoor settings. Outdoor locations include the following: *A. curassavica* from the Gerardo Garden and two different yards in Miami, Florida, and *A. incarnata* from the De Roode Garden and The Carter Center. Each point represents the proportion of leaf pieces containing a cultivable fungal endophyte in one sampled leaf.

The first plants sampled in the study were *Asclepias incarnata* and *A. curassavica* that had been grown in the greenhouse at Emory University's Michael Street parking deck. Greenhouse samples showed a low fungal endophyte infection prevalence (Fig. 1). The mean proportion of infected leaf pieces per *A. incarnata* leaf was 0.04. and the mean proportion of infected leaf pieces per *A. curassavica* leaf was only 0.02. The low rates of fungal endophyte infection in greenhouse plants prompted interest in the collection of *A. incarnata* and *A. curassavica* from natural garden environments for comparison. *A. incarnata* collected from the Carter Center and the De Roode Garden both showed much higher rates of endophytic infection. The mean proportion of infected leaf pieces per *A. incarnata* leaf from the De Roode Garden was 0.53, while the analogous proportion exhibited in Carter Center *A. incarnata* was 0.27. *A. curassavica* taken from natural environments also tended to be more heavily infected with fungal endophytes than samples that had been grown in the greenhouse environment. The mean proportion of

infected leaf pieces per *A. curassavica* leaf sampled from the Gerardo garden was 0.11. Two yards in Miami, Florida were also utilized for *A. curassavica* collection. The mean proportion of infected leaf pieces per Miami Yard A *A. curassavica* leaf was 0.24, while this proportion was 0.09 in *A. curassavica* from Miami Yard B. Overall, while there was no significant influence of plant species on infection prevalence ($X^2 = 1.78$, d.f. = 1, $p = 0.18$) , there was a significant influence of location (greenhouse versus outdoors) ($X^2 = 5.12$, d.f = 1, $p < 0.03$). There was no significant plant species by location interaction ($X^2 = 0.13$, d.f. = 1, $p = 0.72$).

Beyond prevalence (the proportion of leaf pieces infected within leaves) we can also consider incidence (the proportion of leaves infected as indicated by fungus emerging from any sampled leaf piece within a leaf). As shown in Fig. 1, the incidence in fungal endophyte infection in greenhouse plants is fairly low in sampled *A. curassavica* leaves. The majority of greenhouse *A. curassavica* leaf infection proportions are clustered tightly at zero. However, there are three leaf infection proportions which exceed zero, giving a fungal endophyte incidence of three infected leaves out of 15 sampled greenhouse *A. curassavica* leaves. Incidence of fungal endophyte infection is also low in greenhouse *A. incarnata* plants: 5 of 15 sampled greenhouse *A. incarnata* leaves were infected.

Incidence of fungal endophyte infection in outdoor *Asclepias* plants is higher overall than fungal endophyte incidence in the greenhouse *Asclepias* plants (Fig. 1). Out of 17 outdoor *A. curassavica* leaves sampled, 9 leaves showed infection, and 12 of 19 outdoor *A. incarnata* leaves showed fungal endophytic infection. Overall, when pooling together data from both plant species, the incidence of fungal endophyte infection was significantly greater in outdoor settings (21 of 36 leaves) than in the greenhouse (8 of 30 leaves) ($X^2 = 6.66$, d.f. = 1, $p < 0.01$).

Variation in endophytic infection prevalence and incidence among different host plant genera from the same location

Due to difficulties encountered in sampling *Asclepias* species grown in the same environment, we were interested in examining how plants grown in the same location compared in fungal endophytic infection prevalence. To begin to explore this question, we sampled plants from the following genera in the Gerardo Garden: *Asclepias* (milkweed), *Rosa* (rose), *Callicarpa* (American Beauty Berry), and *Vaccinium* (blueberry). An exploratory analysis showed significant differences in fungal endophytic infection prevalence among the four different species of plants despite being collected from an identical location ($X^2 = 12.63$, d.f.= 3, $p < 0.01$) (Fig. 2). Leaf pieces from the *Rosa* genus exhibited the lowest prevalence of fungal endophytic infection. None of the 120 *Rosa* leaf pieces contained cultivable fungal endophytes. Leaf pieces collected from *Asclepias curassavica* were the next lowest in fungal endophytic infection prevalence; the mean proportion of infected leaf pieces per *A. curassavica* leaf was 0.11. *Callicarpa* leaf pieces showed higher prevalence of fungal endophytes, with a mean proportion of infected leaf pieces per *Callicarpa* leaf of 0.29. The highest prevalence of fungal endophytic infection was observed in the *Vaccinium* genus. The mean proportion of infected leaf pieces per leaf from the *Vaccinium* genus was 0.55.

Differences in fungal endophyte infection incidence were also observed among the four species sampled from the Gerardo Garden (Fig. 2). The *Rosa* genus plant had an endophytic incidence of 0, with no leaves exhibiting infection. At three infected leaves out of seven (proportion = 0.43), *A. curassavica* showed a higher incidence of fungal endophyte infection.

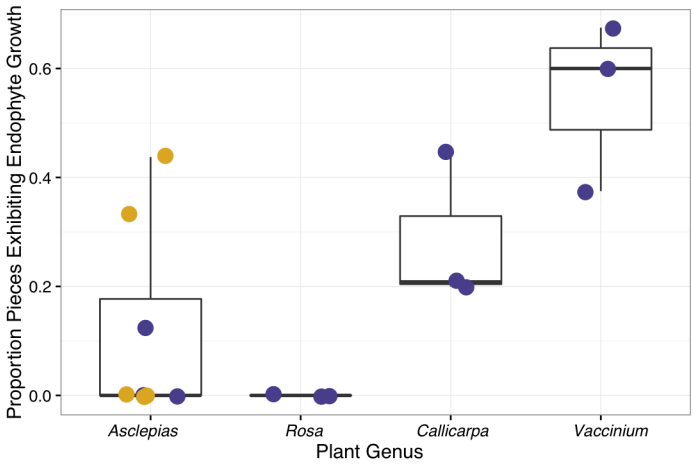


Figure 2: Proportion of leaf pieces exhibiting fungal endophytic infection in four different genera sampled from the Gerardo Garden. Each point represents the proportion of leaf pieces containing a cultivable fungal endophyte in one leaf. *A. curassavica* leaves were taken from two different plants; various colors on *A. curassavica* plot distinguish the plant supplying each leaf. Analysis is exploratory, as only one plant each was used for the collection of *Rosa*, *Callicarpa*, and *Vaccinium* data. Note that incidence data is available, where a proportion of zero would indicate the leaf is uninfected, and a proportion greater than zero would indicate that the leaf is infected.

Host plants from the genera *Callicarpa* and *Vaccinium* showed the highest fungal endophyte infection incidence; all of the leaves sampled from both genera were observed to be infected with fungal endophytes.

Effect of location on fungal endophytic infection prevalence and incidence in *Asclepias*

Prevalence and incidence of fungal endophyte infection in A. incarnata across locations

After collecting data suggesting that different species of plants from the same location had varying fungal endophytic infection prevalence, we were interested in exploring how the same species of plants sampled across different locations compared in infection prevalence. We assessed this question by comparing data, separately, from both *A. incarnata* and *A. curassavica*. The first comparison performed was between *A. incarnata* sampled from The Carter Center and the De Roode Garden (Fig. 3). Fifteen leaves from five *A. incarnata* plants were collected from the Carter Center. These samples exhibited a mean proportion of infected leaf pieces per leaf of 0.27. From the De Roode Garden, four leaves (80 total leaf pieces) were plated and exhibited a

mean proportion of infected leaf pieces per leaf of 0.53. The difference between infection prevalence between the Carter Center and De Roode Garden samples was not statistically significantly different ($X^2 = 1.11$ d.f. = 1 p = 0.29).

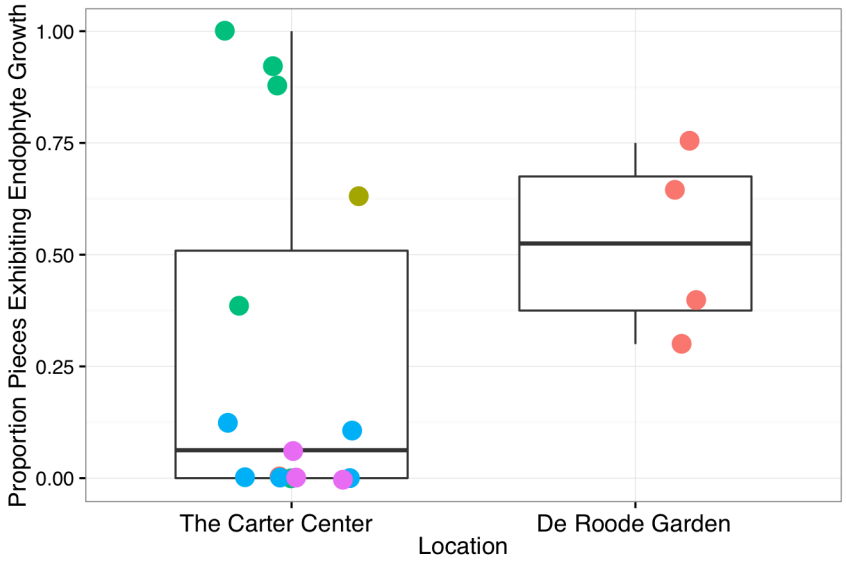


Figure 3: Proportion of leaf pieces exhibiting fungal endophytic infection in *A. incarnata*. Only *A. incarnata* sampled from natural locations are included in this analysis. Each point represents the proportion of leaf pieces containing a cultivable fungal endophyte in one leaf. Colors indicate plant from which each leaf originated.

Furthermore, the De Roode Garden *A. incarnata* demonstrated a higher incidence of infection (4 of 4 leaves sampled were infected) than Carter Center *A. incarnata* (8 leaves of 15 sampled leaves were infected). This difference was not statistically significantly different (Fisher's Exact Test: p = 0.25).

Prevalence and incidence of fungal endophyte infection in *A. curassavica*

We also performed a comparison of fungal endophyte prevalence among *A. curassavica* plants grown in different locations. The comparison included data from *A. curassavica* grown in the Gerardo Garden as well as *A. curassavica* sampled from the yards in Miami, Florida (Miami Yard A and Miami Yard B). Miami Yard B had the lowest prevalence of fungal endophyte infection. The mean proportion of infected leaf pieces per Yard B leaf was 0.09. Leaves taken from the Gerardo Garden had a slightly higher prevalence of endophytic infection, with a mean

proportion of infected leaf pieces per leaf of 0.11. Miami Yard A had the *A. curassavica* samples with the highest fungal endophyte prevalence; the mean proportion of infected leaf pieces per *A. curassavica* leaf from Yard A was 0.24. These differences were not statistically significantly different (X^2 = 3.09, d.f = 2, p = 0.21).

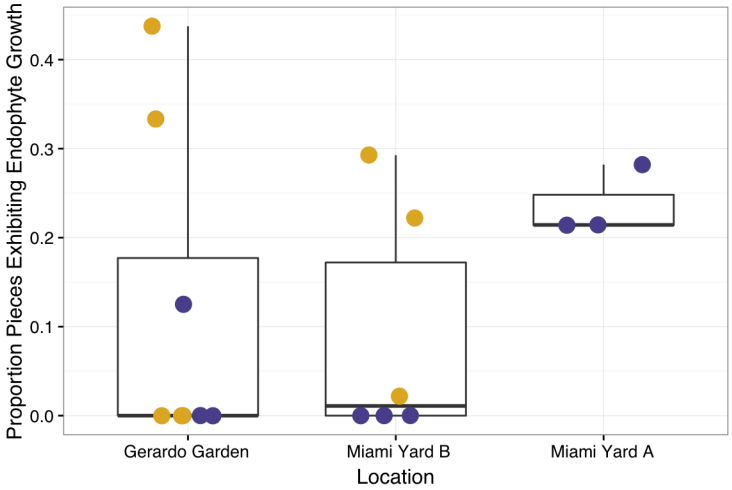


Figure 4: Proportion of leaf pieces exhibiting fungal endophytic infection in *A. curassavica*. Each point represents the proportion of infected leaf pieces in a single *A. curassavica* leaf sampled. With a location, colors indicate the plant from which each leaf originated.

In examining the incidence of fungal endophyte infection across *A. curassavica* taken from the three locations, we also observed differences. The highest incidence of fungal endophyte infection was observed at Miami Yard A at 3 of 3 sampled leaves showing infection. *A. curassavica* taken from the Yard B exhibited the next highest incidence at 3 of 6 sampled leaves showing infection. The lowest incidence of fungal endophytic infection was observed in Gerardo Garden *A. curassavica*, with 3 of 7 sampled leaves showing infection.

Fungal endophyte infection prevalence and incidence compared across four species of *Asclepias*

All *Asclepias* data collected from outdoor locations was compiled to compare fungal endophytic infection prevalence among four species: *A. tuberosa, A. incarnata, A. syriaca,* and *A. curassavica*. The mean proportion of infected leaf pieces per leaf was highest in the sampled *A. tuberosa*, at 0.57. *A. syriaca* leaf pieces were next highest in fungal endophyte infection prevalence; the mean proportion of infected leaf pieces per leaf was 0.42. Next in decreasing

prevalence was *A. incarnata.* Roughly one third (mean proportion = 0.33) of *A. incarnata* leaf pieces were infected per leaf sampled. Fungal endophytes were least prevalent in *A. curassavica*. The mean proportion of infected leaf pieces per *A. curassavica* leaf was 0.13. These differences were not significantly different when taking into account the sampling structure (*i.e.,* the non-independence of leaves sampled within a plant) (X^2 = 6.06, d.f. = 3, p = 0.11).

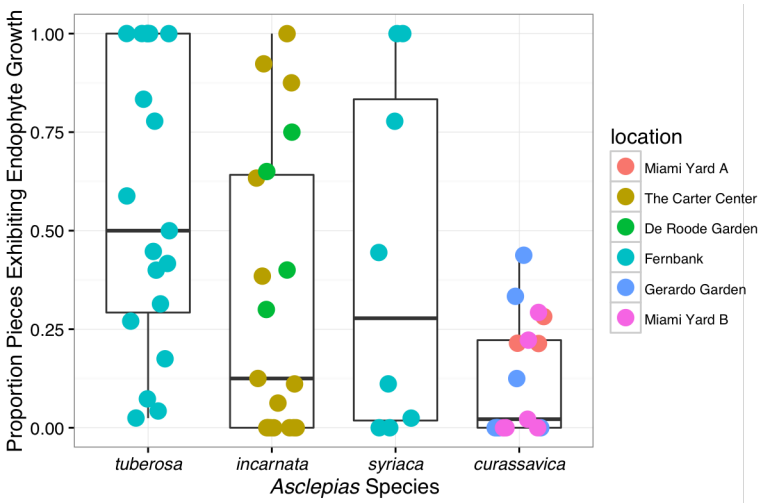


Figure 5: Proportion of leaf pieces exhibiting fungal endophytes across four *Asclepias* species. Each point represents the proportion of infected leaf pieces in one sampled leaf. Color coding indicates locations of origin for each of the leaves. *Asclepias* species are arranged from lowest in cardenolide concentration to highest.

Fungal endophyte incidence varied amongst the four species, with incidence highest in *A. tuberosa*. All 19 leaves sampled from *A. tuberosa* specimens demonstrated fungal endophyte infection. The next highest fungal endophyte incidence was observed in *A. syriaca*, with 6 of the 8 sampled leaves (proportion = 0.75) exhibiting fungal endophyte infection. *A. incarnata* was next in order of decreasing incidence; 12 of 19 sampled leaves (proportion = 0.63) were infected with endophytes. *A. curassavica* demonstrated the lowest incidence of fungal endophyte infection, with only 9 of 17 sampled leaves (proportion = 0.53) indicating infection.

Morphotype classification, identification and assessment of sampling depth

Fungal endophytes from 376 out of 436 infected *Asclepias* leaf pieces were classified into 30 morphotype groups. Of these 30 morphotypes, represented samples of three have thus far been identified to genus using DNA sequencing. Descriptions are available in Table 2.

Using these data, we created a "species" accumulation curve to assess the thoroughness of morphotype sampling (Fig. 6). Here, I will refer to the curve more accurately as a morphotype accumulation curve, as we have not yet resolved the morphotype groups to genera or respective species. In the curve, we see that as the number of samples increases, the slope of the curve begins to decrease. The shape of the morphotype accumulation curve is reminiscent of logarithmic growth; as we move toward more samples, the number of morphotypes discovered increases by fewer morphotypes, suggesting that we have captured much of the cultivable fungal endophtye diversity that was present in these milkweed species at these locations and points in time.

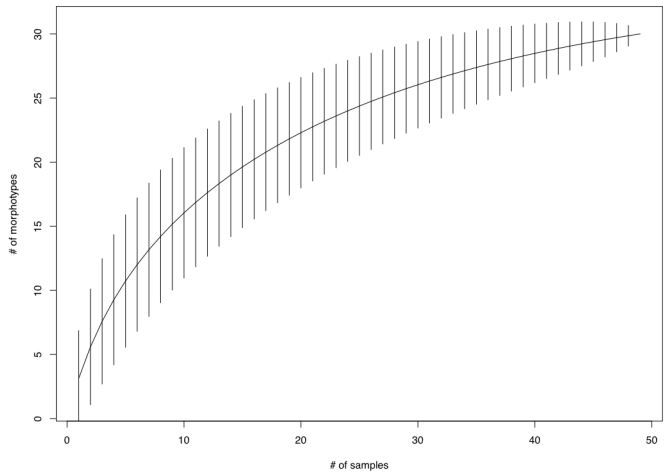


Figure 6: Morphotype accumulation curve.
Fungal endophyte morphotype classification data from 376 *Asclepias* leaf pieces were used to create the curve.

Table 4. Descriptions of Morphotypes. The genus of each morphotype is indicated where a portion of the ITS region of a representative sample has been sequenced. All sequences were greater than 99% identical to multiple reference sequences of that genera within NCBI. n/a = not available. See Appendix C for photographs of each morphotype group.

Morphotype Group	Description	Genus	Cardenolide Zone of Inhibition Assay (T=tested, NT=not tested).
1	Yellow, dusty appearance, prolific.	n/a	NT
2	Black, mottled appearance. Medium density.	n/a	T
3	Black, furry texture. Dense.	n/a	NT
4	Bright pink/orange, moderately dense.	n/a	T
5	Green, fuzzy center with white edges, moderately dense.	n/a	T
6	White, fuzzy texture. Dense.	n/a	NT
7	Black, fuzzy texture. Thin to moderately dense.	n/a	T
8	Orange, sparse. Many dusty spores.	n/a	NT
9	Green with fuzzy white top. Dense.	n/a	NT
10	Black, uneven, dusty texture.	n/a	NT
11	Brown, feathery. Moderately dense.	*Alternaria sp.*	NT
12	Black, granular texture. Moderately dense.	*Xylaria sp.*	NT
13	White, very flat with blotchy appearance.	n/a	NT
14	White, snowflake pattern. Thin to moderately dense.	*Nemania sp.*	T (two samples, 14A & 14B)
15	Uniform green/black and dense.	n/a	T
16	Brown center with white outer ring or top. Fuzzy texture.	n/a	T
17	Green/white and sparse.	n/a	NT
18	White center with green edge. Moderately dense.	n/a	NT

19	Brown/white, star-like growth pattern. Moderately dense.	n/a	T
20	Very dense green, beadlike.	n/a	NT
21	White and black, spotted. Moderately dense.	n/a	T
22	Green with starlike growth pattern. Moderate density.	n/a	T
23	Black, dense growth with white starlike structures.	n/a	NT
24	Orange, very dense, little spread. Discolors agar to intense yellow.	n/a	NT
25	White, moderately sparse fungus with black streaks throughout.	n/a	NT
26	Orange with black spots uniformly distributed. Moderate density.	n/a	T
27	White, very flat or embedded in agar. Slow growth.	n/a	NT
28	White and black fungus with a swirled, truffle-like appearance. Flat and dense.	n/a	NT
29	Orange, beadlike fungus. Dense.	n/a	NT
30	Green/white with spider web appearance. Moderately sparse.	n/a	NT

Influence of *Asclepias* species on fungal endophyte community membership

To begin to compare fungal endophyte communities among the four different *Asclepias* species sampled, we compiled data on the morphotype groups found in association with each host plant species, along with the frequencies at which each morphotype was cultivated. Infected leaf pieces having missing morphotype classification data were excluded from the analyses. Data were used to create a stacked bar plot for each of the *Asclepias* host plant species sampled (Fig. 7).

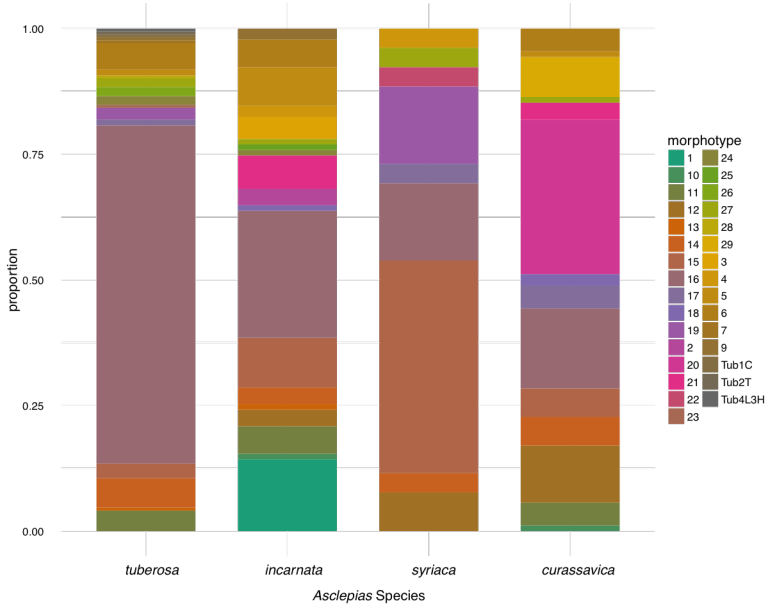


Figure 7: Fungal endophyte community composition by morphotype group for four *Asclepias* species. Proportion here is defined as the number of leaf pieces from each *Asclepias* species infected with an endophyte from a particular morphotype group divided by the number of all infected leaf pieces (identified to morphotype) of that *Asclepias* species. Infected leaf pieces that were not assigned a morphotype group are excluded in the analysis.

The fungal endophytic communities do appear to vary amongst differing host species. The largest component of *A. tuberosa's* fungal endophyte community is fungus belonging to morphotype group 16, followed by group 14 and group 6 fungi. *A. tuberosa* sampled in this study exhibited fungal endophytes falling into 19 different morphotype groups. In *A. incarnata*, group 16 fungi were also found to be the most prevalent component of the host species' endophytic community, though a lower proportion of the *A. incarnata* endophytic community is characterized as group 16 when compared to the *A. tuberosa* endophytic community. The next largest component of the *A. incarnata* fungal endophyte

community is group 1 fungi, followed by the group 5 fungi. As in the *A. tuberosa* endophytic community, we also observed fungal endophytes representing 19 morphotype groups in the *A. incarnata* community.

The *A. syriaca* fungal endophyte community differs from *A. tuberosa* and *A. incarnata* in that its largest component is fungi from morphotype group 15. The next largest components of the *A. syriaca* fungal endophyte community are fungi from groups 19 and 16, respectively. In *A. syriaca,* we observed slightly less diversity in the makeup of the fungal endophytic community, with fungal endophytes cultivated from 9 morphotype groups instead of the 19 morphotype groups observed in *A. tuberosa* and *A. incarnata*.

The *A. curassavica* fungal endophyte community differs from all three of the other sampled *Asclepias* species in that it has a substantial component of fungi from morphotype group 20, which is a relatively minor component in the fungal endophyte communities of *A. tuberosa*, *A. incarnata,* and *A. syriaca*. Next in order of largest components in the *A. curassavica* fungal endophyte community are fungi from group 16, followed by fungi from group 12. We observed slightly higher diversity in the *A. curassavica* fungal endophyte community than in *A. syriaca,* with fungi from 14 different morphotype groups isolated. However, the *A. curassavica* fungal endophyte community was still less diverse than those of *A. tuberosa* and *A. curassavica*, which both had 19 different morphotype groups isolated.

Influence of location on fungal endophyte community membership

Fungal endophyte community comparisons were also performed under conditions in which the host plant species was held constant and the location varied. Both *A. incarnata* and *A. curassavica* were used for these comparisons. *A. incarnata* cuttings sampled from three locations (De Roode Garden, The Carter Center, and Emory's Michael Street Greenhouse) were used in the first comparison (Fig. 8). Differences in fungal endophytic communities were observed despite the leaf pieces originating from the same species (*A. incarnata*). Infected leaf pieces sampled from greenhouse plants had an endophytic community dominated by fungi from morphotype group 1. The next largest component of the greenhouse *A. incarnata* endophytic community were fungi from morphotype group 5, followed by fungi from group 12. Greenhouse *A. incarnata* plants showed the lowest endophytic morphotype diversity of the three locations sampled, with only five morphotype groups cultivated.

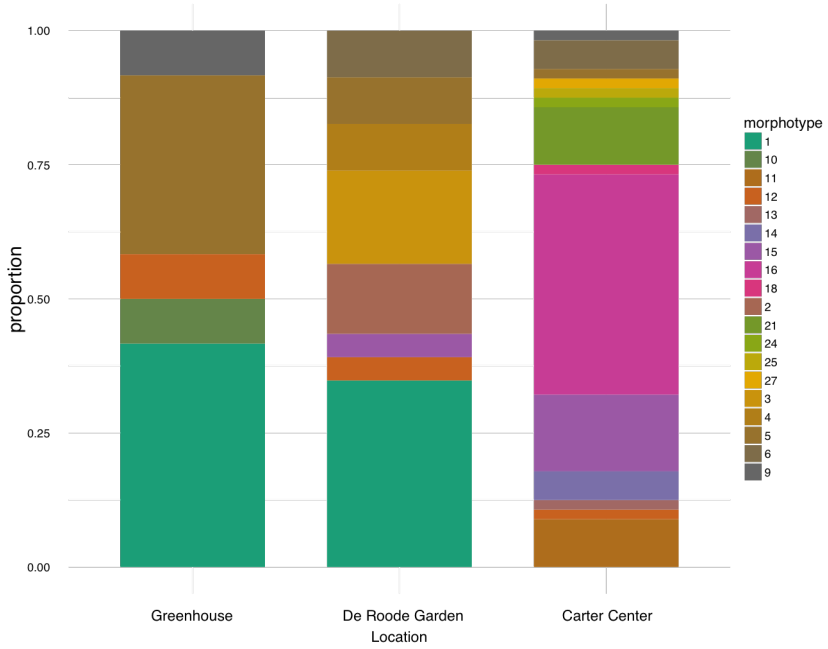


Figure 8: Fungal endophyte community composition by location in *A. incarnata*. Proportion is defined as the number of leaf pieces from each location infected with a fungal endophyte from a particular morphotype group divided by the total number of infected leaf pieces collected from that respective location. Data from infected leaf pieces without morphotype classification is excluded.

A. incarnata sampled from the De Roode Garden showed some differences in its endophytic community when compared to the greenhouse samples, though the most dominant endophyte type was still morphotype group 1. Fungi from morphotype group 3 were the second largest component of the De Roode *A. incarnata* fungal endophyte community, while fungi from morphotype group 2 were the third largest component. The De Roode Garden *A. incarnata* samples had greater fungal endophytic morphotype diversity than the greenhouse *A. incarnata* samples, at eight morphotype groups observed.

The fungal endophytic community observed in The Carter Center leaf pieces was markedly different from the communities observed in both the greenhouse and the De Roode Garden; most notably, The Carter Center *A. incarnata* fungal endophytic community was more diverse, with fungi from 14 different morphotype groups. In addition, fungi from group 1, which had been the largest component of the Greenhouse and De Roode Garden *A. incarnata* fungal endophyte communities, were entirely absent from the infected *A. incarnata* pieces sampled from The Carter Center. The most dominant component of The Carter Center *A. incarnata* fungal endophytic community was fungi of morphotype group 16, a group not represented in the Greenhouse and De Roode Garden *A. incarnata* samples. The second largest component of The Carter Center community was fungi from morphotype group 15; the third largest component was fungi from morphotype group 21.

Comparison across locations was also feasible for *A. curassavica*. The *A. curassavica* comparison used data from plants grown in Emory's Michael Street Greenhouse, the Gerardo Garden, and Yards A and B in Miami, Florida (Fig. 9). The fungal endophytic community of greenhouse *A. curassavica* exhibited very little diversity, with fungi from only two morphotype

groups collected. The largest component of the greenhouse endophytic community was fungi from morphotype group 17, followed by fungi from morphotype group 15.

Gerardo Garden *A. curassavica* samples showed more diversity in their fungal endophytic community than did greenhouse plants. Fungi representing 7 morphotype groups were cultivated from Gerardo Garden leaf pieces. The most dominant member of the Gerardo Garden *A. curassavica* fungal endophyte community was fungi from morphotype group 16, followed by fungi from groups 18 and 21, respectively.

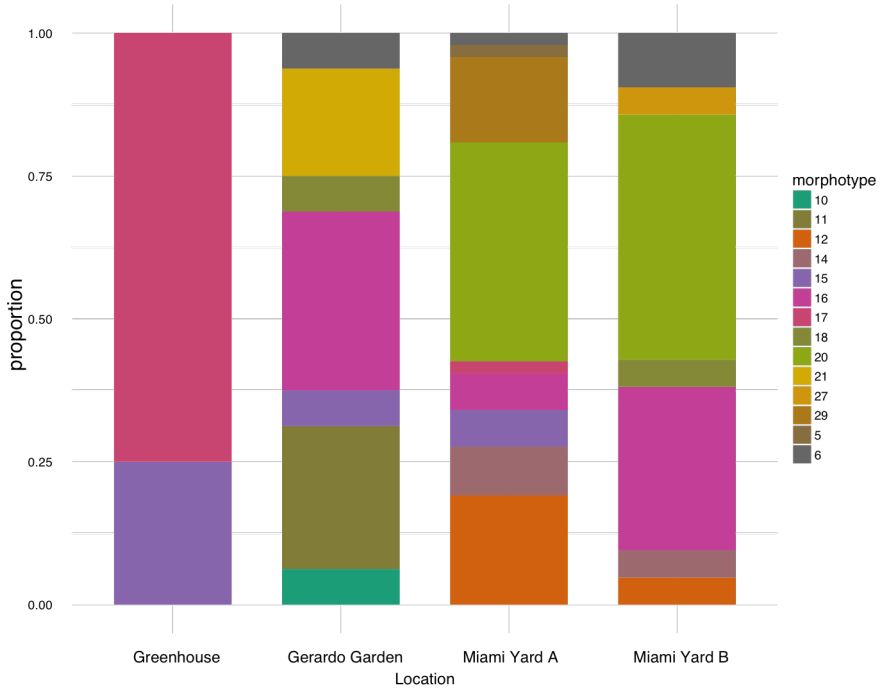


Figure 9: Fungal endophyte community composition by location in *A. curassavica*. Proportion is defined as the number of leaf pieces from each location infected with endophytes of a particular morphotype divided by the total number of infected leaf pieces sampled from that respective location. Data from infected leaf pieces without corresponding morphotype classification was excluded.

Miami Yard A exhibited the greatest diversity in its fungal endophytic community when compared to *A. curassavica* collected from the Greenhouse, the Gerardo Garden, and Miami Yard B. Fungi belonging to 9 morphotype groups were isolated and cultivated from infected leaf pieces from Miami Yard A. The largest component of the Miami Yard A fungal endophyte community was fungi belonging to morphotype group 20, which was represented in neither

greenhouse nor Gerardo Garden *A. curassavica* leaf pieces. The second largest component of the Miami Yard A fungal endophyte community was fungi from morphotype group 12, followed by fungi from group 29.

At seven morphotype groups represented, Miami Yard B had a fungal endophytic community that was more diverse than that of the Greenhouse and Gerardo Garden samples, but still less diverse than that of Miami Yard A. However, the Miami Yard B fungal endophytic community was similar to the Miami Yard A community in that the most dominant member was fungi from morphotype group 20. The next largest component of the Miami Yard B *A. curassavica* fungal endophytic community was fungi from morphotype group 16, followed by fungi from group 6.

Influence of *Asclepias* species on fungal endophyte community richness and composition

The number of different fungal endophyte morphotyes found in each leaf sampled did not vary substantially between plant species (Fig. 10). *A. curassavica* had the highest average number of morphotypes isolated from each leaf, with a single *A. curassavica* leaf containing fungal endophytes belonging to four different morphotype groups on average. *A. syriaca* had the next highest average number of fungal endophyte morphotypes isolated from each leaf; the typical *A. syriaca* leaf contained fungal endophytes belonging to ~3-4 morphotype groups. *A. tuberosa* was next in order of decreasing average number of fungal endophyte morphotypes isolated per leaf; the average *A. tuberosa* leaf was infected with fungal endophytes belonging to

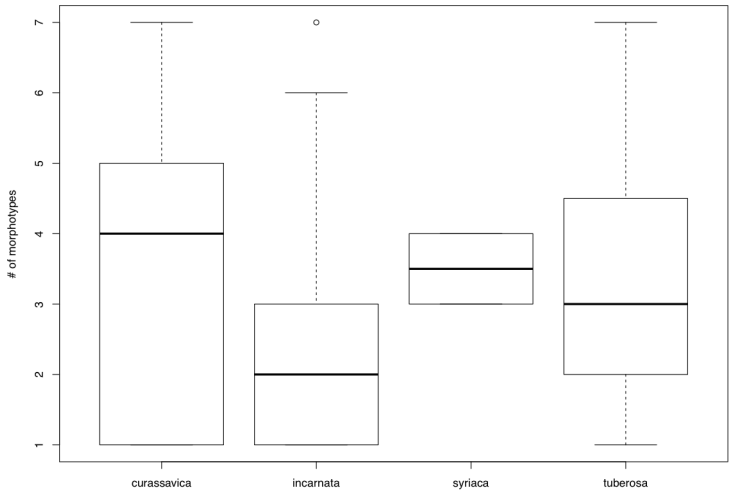


Figure 10: Fungal endophyte morphotypes by *Asclepias* species. Boxplots were assembled using data from the number of different fungal endophyte morphotypes collected from each leaf sampled. Middle bar in each boxplot represents the median number of morphotypes collected per leaf of the corresponding species.

three different morphotype groups. *A. incarnata* showed the lowest average number of morphotypes collected per leaf. A typical *A. incarnata* leaf was infected with fungal endophytes belonging to two different morphotype groups.

Using morphotype group data from each *Asclepias* leaf sampled, a cluster dendogram was created to highlight any

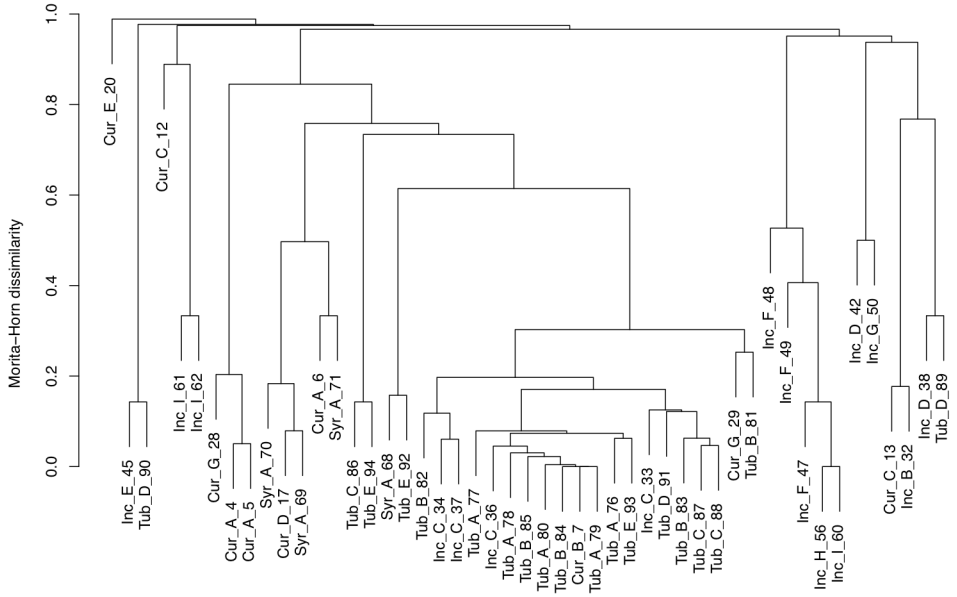


Figure 11: Cluster dendogram of sampled *Asclepias* leaves based on Morista-Horn Dissimilarity Indices. There is no obvious clustering by plant species. Each sample code represents plant species (first three letters), plant (fourth letter), and leaf number. Cur = *A. curassavica*, Inc = *A. incarnata*, Tub = *A. tuberosa*, Syr = *A. syriaca.*

similarities in fungal endophyte morphotype makeup among the different leaves (Fig. 11). No substantial clustering by host plant species is apparent. There are several instances of leaves taken from the same plant creating pairs and small clusters on the dendogram, but we also observe small clusters of leaves that are from different plants and even different species. An NMDS plot similarly indicates little clustering based on *Asclepias* spp. (Fig. 12). Community composition did not significantly differ between the plant species when taking into account the non-independence of leaf samples from the same plant (permutational multivariate analysis of variance: $r^2 = 0.21$, d.f. = 3, p = 1).

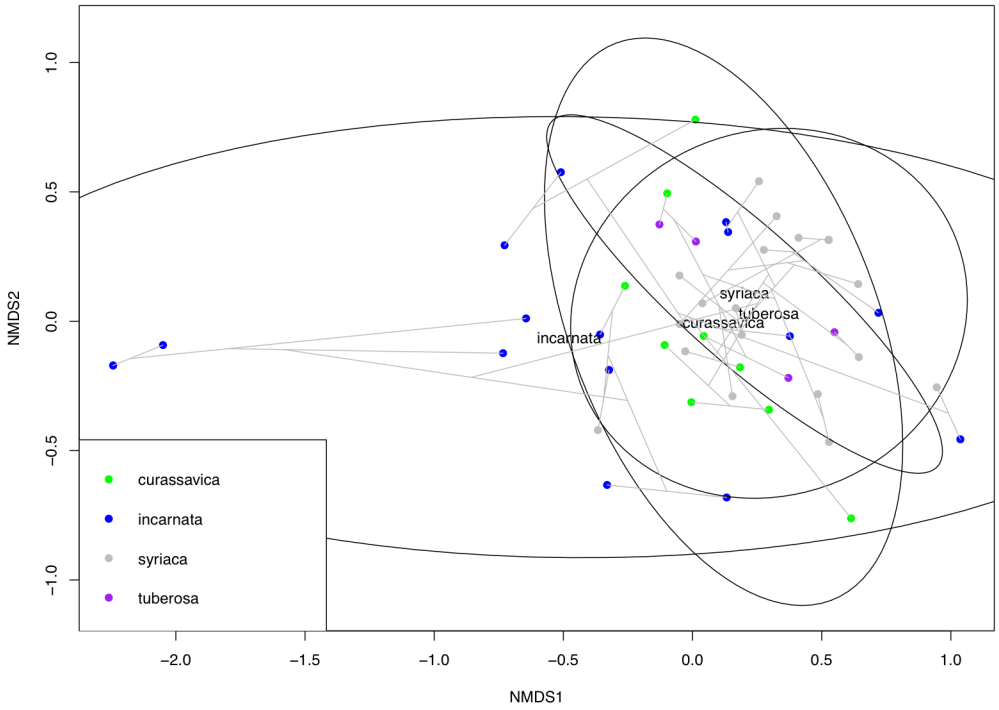


Figure 12: NMDS plot of *Asclepias* fungal endophyte morphotype composition data. We do not see substantial separate clustering of samples from the four species within the NMDS plot, further suggesting no significant differences in fungal endophyte morphotype compositions in the leaves of the four species sampled.

Latex zone of inhibition assays

Preliminary results examined as images of the zone of inhibition plates suggested that there was no appreciable difference in fungal endophyte growth in the presence of milkweed latex, regardless of the donor *Asclepias* plant from which the latex had been derived (Fig. 13). This appeared true for all fungal endophytes tested.

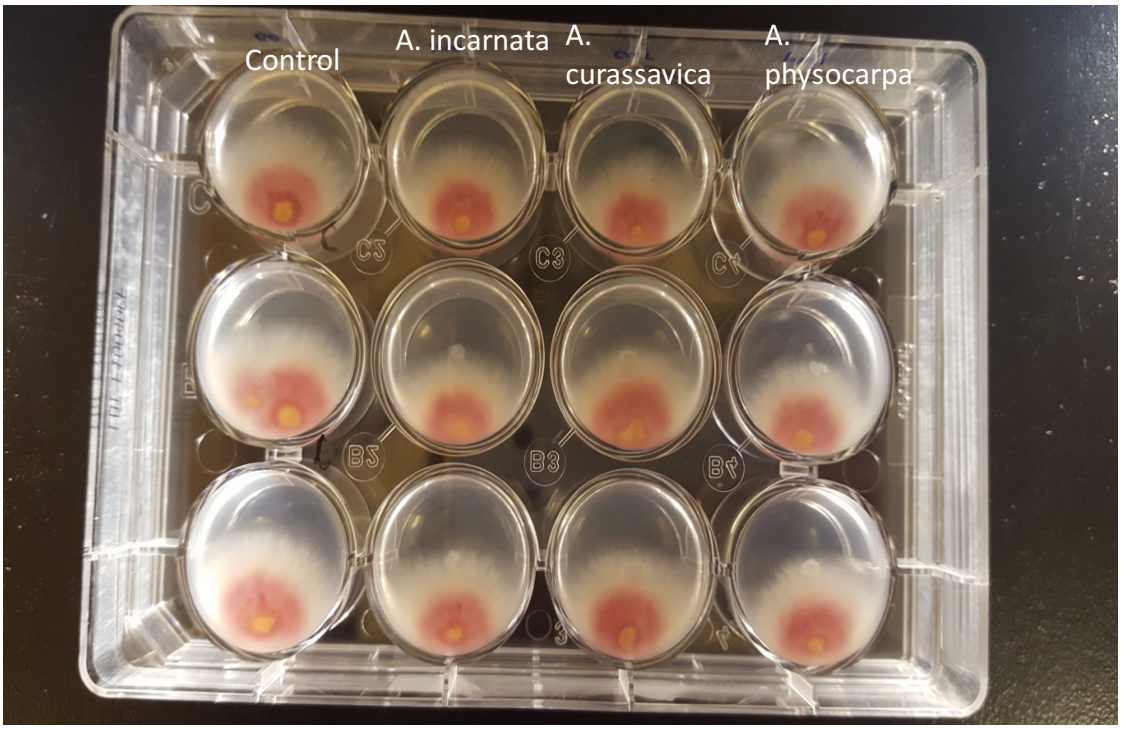


Figure 13: Fungal endophyte morphotype group 26 zone of inhibition assay, day 3. Fungi from morphotype group 26 did not show appreciable inhibition of growth in the presence of latex. Similar results were seen in assays with eleven other fungal endophytes from different morophotypes.

However, when relative growth data was tabulated for the assays, a general trend suggesting less fungal growth in latex test wells became apparent (Fig. 14). In each of the latex treatment groups, several relative growth measurements fell below the control value of 1, suggesting that the presence of latex may slightly inhibit fungal endophyte growth. Figure 14 demonstrates similar scatter of relative growth measurements among latex test wells for each of the *Asclepias* host plants used, despite *A. incarnata, A. curassavica,* and *A. physocarpa* having

varying cardenolide concentrations. As a result, it is unclear whether the slight apparent inhibitory effect of latex is related to or caused by cardenolides within the latex.

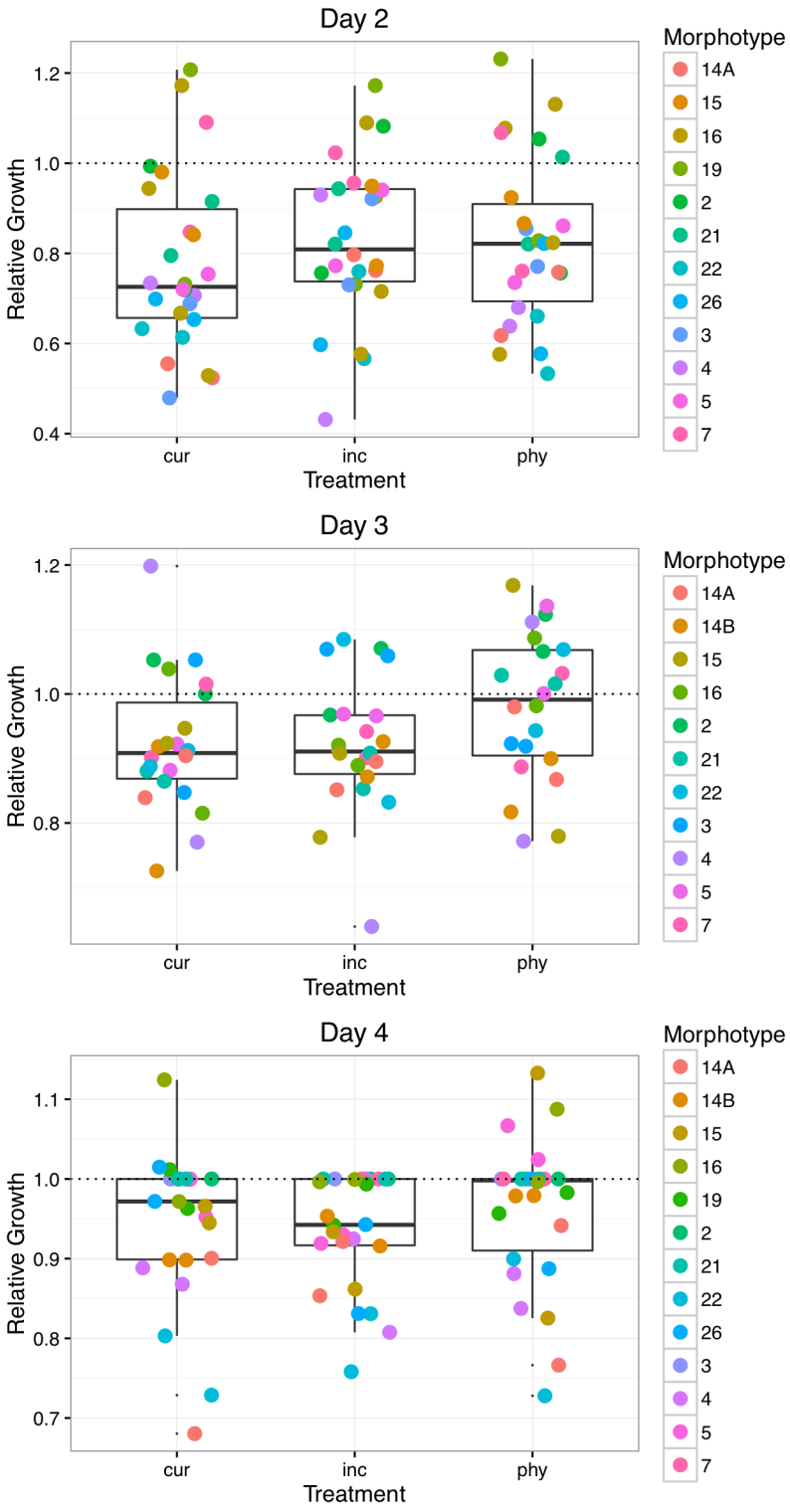


Figure 14: Zone of inhibition assay results. Colors of points correspond to the morphotype group to which a fungal endophyte belongs and relative growth is reported as the greatest length of fungal growth in the treatment well divided by the greatest length of fungal growth within the latex-free control well. Treatment codes are as follows: cur=*A. curassavica*, inc=*A. incarnata*, and phy=*A. physocarpa*. Note that color assigned to each morphotype group differs slightly among the three graphs.

DISCUSSION

Overall, we find that *Asclepias* plants are host to a wide variety of fungal endophytes—every species examined was host to fungi of several different morphotypes. Host plants grown in natural outdoors environments had, on average, a much higher prevalence and incidence of fungal endophyte infection. We also saw some differences in prevalence and incidence of fungal endophytes when comparing plants of identical species grown in different locations. Our comparison of fungal endophytes among the four species indicate a trend suggesting that high cardenolide species (*A. curassavica*) tend to have lower fungal endophyte prevalence and incidence than low cardenolide species, such as *A. tuberosa*. However, statistical analysis shows that this trend is not statistically significant. In analyzing morphotype data from the fungal endophytes collected, we see that there are differences in the fungal endophytes isolated from plants of different species, as well as *Asclepias* plants of the same species isolated from different locations. Taken together, these results indicate that the fungal endophyte communities in *Asclepias* plants, as well as the factors that shape them, are extremely complex.

Location: greenhouse vs. natural environment

A striking finding from the first phase of the study involved the very low fungal endophyte infection prevalence in greenhouse grown plants of *A. incarnata* and *A. curassavica*. We observed fungal endophyte infection in only 3% of greenhouse *A. incarnata* leaf pieces, while only 2% of greenhouse *A. curassavica* leaf pieces were infected with fungal endophytes. The low prevalence of fungal endophytic infection in greenhouse reared plants is, however, unsurprising when considering the chief sources of fungal endophyte colonization in host plants. Previous studies have noted wind and rain as major vectors for fungal endophyte spores

(Rodriguez et al. 2009, Herre et al. 2005). Specifically, rain and wind play important roles as physical perturbations that aid in releasing and spreading fungal endophyte spores and/or hyphae fragments (Rodriguez et al. 2009). One common method of fungal endophyte colonization involves airborne fungal spores settling on leaf surfaces, followed by the wetting of the leaf tissue. Fungal spores may then germinate under these moist conditions and move into host plant tissue via the stomata (Arnold et al. 2003). Another less commonly known mechanism of fungal endophyte spore introduction involves mycophagy, typically through insects feeding on plant material that contains fungal spores or other fungal material. It has been shown, for example, that fungal spores ingested by grasshoppers may be transmitted through frass in an intact and viable state (Monk & Samuels, 1990). Given these transmission mechanisms, the greenhouse environment may act as a barrier that prevents plants from acquiring fungal endophytes due to its isolation of plant tissue from the natural environment. Plants grown in Emory's Michael Street greenhouse had little exposure to wind and no exposure to rain, both of which are conducive to fungal endophyte infection and spread. In addition, the greenhouse environment was continually monitored for plant pests—plants grown in the greenhouse would have had little if any exposure to insects and their frass, which, as previously mentioned, can act as vectors for fungal endophytes.

Fungal endophytes in *Asclepias* versus other plant genera

The analysis of prevalence and incidence of fungal endophytes in plants from the Gerardo Garden indicated *Asclepias curassavica* had a moderate infection prevalence relative to nearby plants of three other genera. Although each host plant was grown in the same location, fungal endophyte prevalence was significantly different among the species collected. Previous

research has suggested that fungal endophytes can indeed have some degree of host plant specificity, which could in turn influence variation in prevalence within a site. In one study, aspen and beech branches collected from the same location demonstrated differences in fungal endophyte prevalence; a significantly greater proportion of aspen tissue samples were infected with fungal endophytes in comparison to beech tissue samples (Chapela, 1989). Another study demonstrated that mistletoe and fir host plants growing approximately one centimeter apart produced a fungal endophyte community overlap of less than 15% (Petrini et al. 1992). Chemical composition of the host plant is a commonly posed explanation of these fungal endophyte differences. One study demonstrated that *Lolium perenne* (ryegrass) bred to have a higher sugar content is host to a substantially lower concentration of endophytic fungus than *L. perenne* of typical carbohydrate content. In addition, *L. perenne* specimens supplied with extra nitrogen were less heavily infected with endophytes (Rasmussen et al. 2006). Another study noted that the compound camptothecin, a common defense chemical found in plants, kills endophytic fungi unless the fungi have some mechanism to resist the compound (Kusari et al. 2012). Host plants taken from the Gerardo Garden study were sourced from completely different genera, making it likely that the samples had nutrient and chemical profiles that differed from each other. It is possible that some of these host species have profiles that are more conducive to fungal endophyte infection than others, leading to the differences that were observed in fungal endophyte prevalence and incidence.

Fungal endophyte variation by location

Several comparisons across locations were made using data collected in terms of both fungal endophyte prevalence and absence and the makeup of the fungal endophyte communities

in *A. incarnata* and *A. curassavica*. Results showed that even within the same plant species, there was variation in prevalence, in incidence, and in community composition in both *A. curassavica* and *A. incarnata* collected from different locations. Though these differences are not statistically significant, here we explore possible causes behind the phenomenon.

Several studies have indicated different fungal endophytes are found not only amongst different species, but even in the same plant. Woody angiosperms grown in tropical locations, for example, exhibit tremendous fungal diversity, as do many plants found in the temperate zone (Arnold et al. 2003, Arnold et al. 2000). In a study of plants grown in a forest of Panama, 418 morphospecies of fungal endophytes were recovered, but only 140 of these morphospecies were isolated from more than one leaf (Arnold et al. 2000). Fungal endophyte infection in angiosperms occurs via horizontal transmission (Arnold & Herre, 2003), which may explain some of the inconsistencies of fungal endophyte infection and community composition among different plants and even among the leaves of the same plant. Though this project sampled *A. curassavica* and *A. incarnata* plants from different locations (all *A. incarnata* samples were collected in Atlanta, GA while *A. curassavica* were collected from both Florida and Georgia), we must also consider the microenvironments from which the leaves were sampled. Previous studies have demonstrated that individual plants may act as separate ecosystems for fungal endophytes (Petrini et al. 1992). Needles from alpine trees have shown different fungal endophyte prevalence and community compositions based on their positional location within a tree top. Alpine tissues also exhibit lower fungal endophytic infection density as a result of increased exposure to wind (Petrini et al. 1992). The *Asclepias* leaves sampled, though from the same region, almost certainly were grown in different microenvironments—the various collection locations may have provided different amounts of exposure to wind, sunlight, rain, and a number

of other climate related elements. We can expect that the diverse array of microenvironments that were undoubtedly sampled played some role in producing the result that the same *Asclepias* species collected from various sites harbors differences in fungal endophyte infection density and community composition.

Though it is somewhat more common to find differences in environment cited as the cause behind differences in fungal endophytic infection rates and community composition, we can return to a previously cited study—that of the grasshopper and mycophagy—to consider another interesting possibility behind the observed results. Grasshoppers consume spores of fungal endophytes along with their leaf diet, and have been shown to be possible vectors of fungal endophytes, as spores are able to pass through their digestive tracts and emerge viable in frass (Monk & Samuels, 1990). If insects indeed have the ability to be fungal endophyte vectors, then it is no surprise that different locations, which likely provide differing amounts of exposure to insects, may affect both prevalence and community composition of fungal endophytes in host plants. Though we did not consider insect or microenvironment data, considering these variables in future experiments may be helpful in further understanding the factors that moderate fungal endophyte infection.

Fungal endophyte variation in *A. tuberosa, A. incarnata, A. syriaca, and A. curassavica*

Here, we return to the biggest question addressed in this thesis project: do different species of *Asclepias* demonstrate differences in fungal endophyte prevalence and community composition? Referring to Fig. 5 and Fig. 7, we see that there is some suggestion that the four *Asclepias* species tested do vary in endophyte prevalence and community makeup. Statistical analysis of the prevalence data collected among the four species showed that when leaf pieces

were considered independently, the fungal endophyte prevalence difference among the four species was statistically significant (results not shown). However, when we accounted for the fact that leaf pieces were not independent but clustered within leaves, the differences in fungal endophyte prevalence were no longer statistically significant. Likewise, statistical analysis of morphotype data showed that the difference in fungal endophyte community composition among the four *Asclepias* species sampled was not statistically significant when taking into account the non-independence of leaf samples. Further sampling should involve sampling more plants, as this may provide more power to assess the influence of host plant species on fungal endophytes in *Asclepias.*

Though we do not see statistically significant differences, it may be beneficial for future studies to explore causes behind the variability we observed. In explaining the variations in endophyte prevalence and makeup, we must first turn our attention to the fact that the *Asclepias* host plants were, as previously mentioned, collected from different locations, and, in fact, two species (*A. tuberosa* and *A. syriaca*) were only available at a site where the other two species (*A. incarnata*, *A. curassavica*) were not available. Different locations potentially provide different amounts of exposure to natural elements, many of which act as vectors in fungal endophyte infection. Thus, we cannot overlook the possibility that variation in fungal endophyte infection prevalence and community composition that we observed among the four *Asclepias* species may partially be the result of collecting host plants from an array of locations.

Beyond the effect of location, we must consider the effect of chemical composition of the four plant species studied. Plants of the *Asclepias* genus are well-known for their cardenolide content. Certain species of *Asclepias* plants have been demonstrated to harbor higher concentrations of cardenolide compounds, making these species more toxic to animal life

(Sternberg et al. 2012). Previous studies have examined the cardenolide content of several different *Asclepias* species (Agrawal et al. 2008) and noted tremendous variation amongst the species. Using this cardenolide concentration data, we ordered the four *Asclepias* species sampled in this study from highest to lowest cardenolide concentration as follows: *A. curassavica, A. syriaca*, *A. incarnata*, and *A. tuberosa* (Agrawal et al. 2008; Sternberg et al. 2012). Keeping this cardenolide concentration pattern in mind, we find an interesting trend when we examine the fungal endophyte prevalence data (Fig. 5). Leaves sampled from *A. curassavica*, the species with the highest cardenolide concentration, have a dramatically lower median proportion of infected leaf pieces per leaf than do leaves sampled from species with lower cardenolide concentrations, such as *A. tuberosa* and *A. incarnata*. In addition, incidence data indicated that, at an infected leaf proportion of 0.53, the collected *A. curassavica* had the lowest incidence of fungal endophyte infection of the four species sampled. These results prompted us to question the possibility of cardenolide compounds playing some role in fungal endophyte prevalence and incidence among the *Asclepias* host plants. We addressed this question via performing fungal zone of inhibition experiments with several endophytes collected from all four *Asclepias* species.

Images examined from zone of inhibition assays did not show appreciable differences in fungal endophyte growth in the presence of milkweed from any of the three *Asclepias* donor species. However, when relative measurements for fungal endophyte growth were compiled and graphed (Fig. 14), a trend of lower relative growth measurements in latex treatment wells was observed. Though latex treatment wells generally demonstrate slightly lower relative growth measurements, there was no obvious difference in relative growth measurements among the three treatment groups. We are therefore unable to conclude whether any differences in fungal

endophyte growth are the result of cardenolide content within the latex. It is important to note that difficulties were encountered during the preparation of the assay plates, which may in part explain the lack of difference in relative growth measurements among the three treatment groups. The *A. physocarpa* and *A. incarnata* plants used as milkweed donors produced very small amounts of latex, raising the possibility that a slightly smaller amount of latex was used in *A. physocarpa* and *A. incarnata* test wells. Further testing will likely need to be completed to determine whether there is an appreciable inhibitory interaction between milkweed latex (cardenolides) and fungal endophytes.

When we take into consideration the differences in environment and chemical composition of the *Asclepias* plants sampled, it is rather unsurprising that the plants collected exhibited variation in fungal endophyte prevalence and community composition. Due to the design and sampling constraints of this project, it is not possible to definitively discern whether different species of milkweed do in fact harbor statistically significant differences in fungal endophyte infection abundance and community composition; however, our results do shed light on the remarkable intricacy and complexity of the factors which mediate fungal endophyte infection in host plants, and for the first time begin to explore the diverse microbial communities within milkweed leaves.

Considering Diversity of Fungal Endophytes within *Asclepias* Compared to Other Plants

Both results from fungal endophyte morphotype classification (Table 4) and morphotype richness by leaf (Fig. 10) suggested that *Asclepias* plants are host to a diverse array of fungal endophytes. This study sampled 23 host *Asclepias* plants total and found 30 different fungal endophyte morphotypes. However, this degree of fungal endophyte diversity is not particularly

surprising when considering fungal endophyte diversity data from other studies. One study examined the fungal endophyte diversity found in the carnivorous plant genus *Sarracenia*. A host plant representing the *S. purpurea* species was found to have a near 100% fungal endophyte infection prevalence within plant pieces sampled; of the fungal isolates collected from this species, no isolate was found more than once and none of the collected isolates were found in the other *Sarracenia* species tested (Glenn & Bodri, 2012). Similarly, a study examining fungal endophyte diversity within the host plant *Cupressus arizonica* resulted in the collection of 12 different fungal endophyte species (Arnold, 2007). Research aimed at examining the cause of oak forest decline in Europe isolated 210 fungi from the oak (*Quercus)* genus (Moricca & Ragazzi, 2008). Our results then, which suggested appreciable diversity in the *Asclepias* fungal endophytic community, are in agreement with the findings of several previous studies which show tremendous fungal endophyte diversity within host plants of other genera.

In regard to fungal endophytic diversity, an especially striking finding in this project was the remarkable variance in prevalence and fungal endophyte community composition not only at the plant level, but between and within leaves. In Fig. 5, we observe a great deal of scattering of data points, indicating that the fungal endophyte infection prevalence varied dramatically from leaf to leaf, even within the same plant. In addition, in Fig. 10, we observe that for the *Asclepias* plants sampled, each leaf was infected with a median of anywhere from 2-4 different fungal endophyte morphotype groups. Few studies have examined differences in fungal endophyte prevalence among leaves of the same plant. However, one study which examined fungal endophytes in the plant *Coffea arabica* found differences in the fungal endophyte community composition of individual leaves (Santamaria & Bayman, 2005). Further research including

study designs which compare fungal endophyte prevalence and community composition among leaves of the other host plants is necessary to fully place our *Asclepias* data in context.

Future Directions

Additional sampling is need to fully elucidate the influence of location and host plant species on *Asclepias*-associated fungal endophytes. Future steps should include repeating the methods shown with the same host plants sampled across multiple locations. Leaf age has also been shown to be an important determinant in fungal endophyte infection density (Arnold et al. 2000); ensuring even collections of mature and young leaves may be an important consideration in the future. Furthermore, though DNA extraction, PCR, gel electrophoresis, and subsequent sequencing has been completed for a small subset of the fungal endophytes collected, the vast majority of isolates and morphotype groups have not yet been resolved into fungal species. Elucidating the species classification of the endophytes collected will allow us to confirm that fungal isolates within each morphotype group represent the same or similar species.

This project only assesses the prevalence, incidence and diversity of cultivable fungal foliar endophytes. Recently developed approaches to conduct deep sequencing of fungal communities within tissues are just being implemented to quantify and describe fungal foliar endophytes (Lindahl et al. 2013, Siddique & Unterseher, 2016, Tian et al. 2015). These methods could be used in the future to capture additional fungal diversity associated with *Asclepias.*

Finally, milkweeds are host to an array of insect herbivores, many of which may be consuming these fungi within these leaves as they eat the plant tissue. Little is known about how these fungi shape the gut microbiomes or ecologies of these insects. Studies examining *Asclepias* herbivore ecology or disease resistance will need to take into consideration the low fungal

endophyte infection prevalence in greenhouse-raised plants and how this relative absence of fungal endophytes may affect *Asclepia*s herbivores after consumption of the plant.

Conclusions

Through sampling of plant tissues from four *Asclepias* species across multiple locations, we revealed a complex fungal foliar endophyte community associated with milkweed host plants. We found evidence that *A. incarnata* and *A. curassavica* leaves grown in natural outdoors environments have a statistically significantly higher prevalence of fungal endophyte infection than leaves taken from greenhouse plants, which has implications for future experiments using these plants. Furthermore, location may shape these fungal associations; *A. incarnata* and *A. curassavica* leaves taken from host plants in separate locations had varying fungal endophyte infection prevalence as well as different fungal morphotype community compositions. In both species, we find that the difference in both prevalence and community composition data are not statistically significantly different, and further sampling is needed. In examining fungal endophyte infection across *A. tuberosa, A. incarnata, A. syriaca,* and *A. curassavica* host plant species, which differ substantially in their toxic cardenolide composition, we find some differences in fungal endophyte prevalence and community compositions, though these differences are also not statistically significant, and further sampling is again needed. It is possible that more thorough sampling may add sufficient power to the study to demonstrate statistically significant differences among the *Asclepias* species. More specifically, sampling a greater number of leaves from more plants within each species may help to elucidate whether there are differences in the four species' fungal endophytic communities, and whether these differences are tied to the chemical composition of these chemically diverse, toxic plants.

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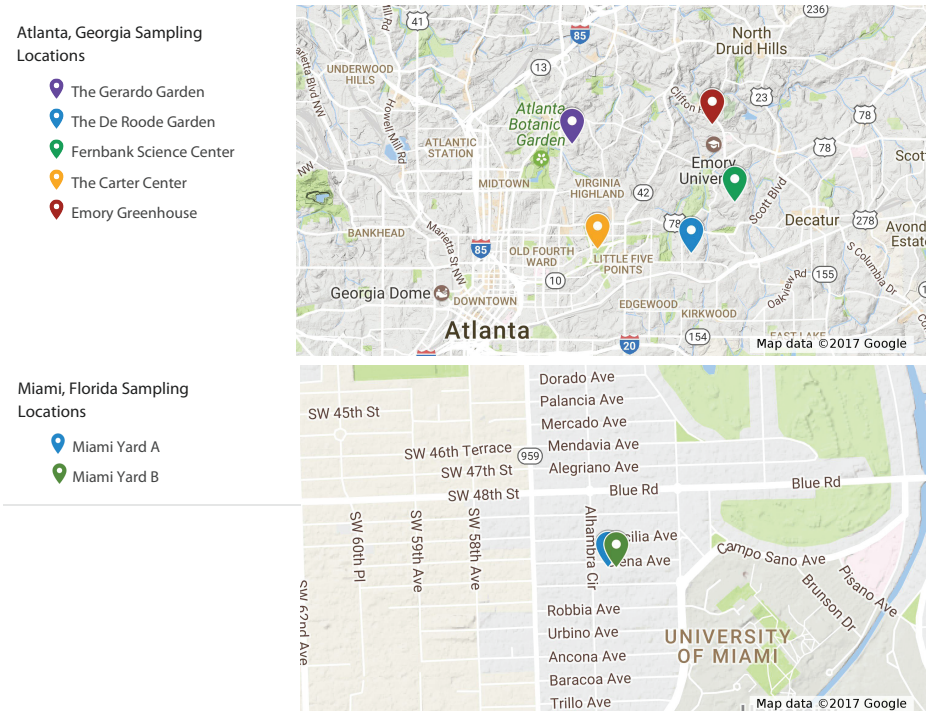
Appendix A

Maps of Host Plant Collection Locations



Sample collection sites.

Appendix B

Sampling Tables

Table 2: Samples collected by plant.

Plant Genus	Plant Species	Location	Plant	Total Pieces	No. of Infected Pieces	No. of Infected Pieces Classified to Morphotype Group
Asclepias	*curassavica*	Miami Yard A	Cur A	226	51	47
Asclepias	*curassavica*	Gerardo Garden	Cur B	120	5	5
Asclepias	*curassavica*	Gerardo Garden	Cur C	77	11	11
Asclepias	*curassavica*	Greenhouse	Cur D	145	1	1
Asclepias	*curassavica*	Greenhouse	Cur E	145	4	3
Asclepias	*curassavica*	Miami Yard B	Cur F	114	0	0
Asclepias	*curassavica*	Miami Yard B	Cur G	132	23	21
Asclepias	*incarnata*	Carter Center	Inc A	39	0	0
Asclepias	*incarnata*	Carter Center	Inc B	30	19	9
Asclepias	*incarnata*	Carter Center	Inc C	71	44	43
Asclepias	*incarnata*	Carter Center	Inc D	73	3	3
Asclepias	*incarnata*	Carter Center	Inc E	48	1	1
Asclepias	*incarnata*	De Roode Yard	Inc F	80	42	23
Asclepias	*incarnata*	Greenhouse	Inc G	100	2	2
Asclepias	*incarnata*	Greenhouse	Inc H	145	2	2
Asclepias	*incarnata*	Greenhouse	Inc I	134	9	8
Asclepias	*syriaca*	Fernbank	Syr A	41	26	25
Asclepias	*syriaca*	Fernbank	Syr B	137	1	1
Asclepias	*tuberosa*	Fernbank	Tub A	73	71	71
Asclepias	*tuberosa*	Fernbank	Tub B	50	36	36
Asclepias	*tuberosa*	Fernbank	Tub C	112	44	33
Asclepias	*tuberosa*	Fernbank	Tub D	123	19	16
Asclepias	*tuberosa*	Fernbank	Tub E	113	22	15

Callicarpa	*sp.*	Gerardo Garden	Cal A	104	31	0
Rosa	*sp.*	Gerardo Garden	Ros A	120	0	0
Vaccinium	*sp.*	Gerardo Garden	Vac A	120	66	0

Table 3: Samples collected by leaf.

Plant Genus	Plant Species	Location	Plant	Leaf	Total Pieces	No. of Infected Pieces	No. of Infected Pieces Classed to Morphotype Group
Asclepias	*curassavica*	Miami Yard A	Cur A	4	145	31	30
Asclepias	*curassavica*	Miami Yard A	Cur A	5	42	9	9
Asclepias	*curassavica*	Miami Yard A	Cur A	6	39	11	8
Asclepias	*curassavica*	Gerardo Garden	Cur B	7	40	5	5
Asclepias	*curassavica*	Gerardo Garden	Cur B	8	40	0	0
Asclepias	*curassavica*	Gerardo Garden	Cur B	9	40	0	0
Asclepias	*curassavica*	Gerardo Garden	Cur C	10	17	0	0
Asclepias	*curassavica*	Gerardo Garden	Cur C	11	16	0	0
Asclepias	*curassavica*	Gerardo Garden	Cur C	12	12	4	4
Asclepias	*curassavica*	Gerardo Garden	Cur C	13	16	7	7
Asclepias	*curassavica*	Gerardo Garden	Cur C	14	16	0	0
Asclepias	*curassavica*	Greenhouse	Cur D	15	30	0	0
Asclepias	*curassavica*	Greenhouse	Cur D	16	30	0	0
Asclepias	*curassavica*	Greenhouse	Cur D	17	25	1	1
Asclepias	*curassavica*	Greenhouse	Cur D	18	30	0	0

Asclepias	*curassavica*	Greenhouse	Cur D	19	30	0	0
Asclepias	*curassavica*	Greenhouse	Cur E	20	30	3	3
Asclepias	*curassavica*	Greenhouse	Cur E	21	30	0	0
Asclepias	*curassavica*	Greenhouse	Cur E	22	30	0	0
Asclepias	*curassavica*	Greenhouse	Cur E	23	30	0	0
Asclepias	*curassavica*	Greenhouse	Cur E	24	25	1	0
Asclepias	*curassavica*	Miami Yard B	Cur F	25	40	0	0
Asclepias	*curassavica*	Miami Yard B	Cur F	26	44	0	0
Asclepias	*curassavica*	Miami Yard B	Cur F	27	30	0	0
Asclepias	*curassavica*	Miami Yard B	Cur G	28	45	10	10
Asclepias	*curassavica*	Miami Yard B	Cur G	29	41	12	11
Asclepias	*curassavica*	Miami Yard B	Cur G	30	46	1	0
Asclepias	*incarnata*	Carter Center	Inc A	31	39	0	0
Asclepias	*incarnata*	Carter Center	Inc B	32	30	19	9
Asclepias	*incarnata*	Carter Center	Inc C	33	16	14	13
Asclepias	*incarnata*	Carter Center	Inc C	34	13	12	12
Asclepias	*incarnata*	Carter Center	Inc C	35	16	0	0
Asclepias	*incarnata*	Carter Center	Inc C	36	13	5	5
Asclepias	*incarnata*	Carter Center	Inc C	37	13	13	13
Asclepias	*incarnata*	Carter Center	Inc D	38	9	1	1
Asclepias	*incarnata*	Carter Center	Inc D	39	16	0	0
Asclepias	*incarnata*	Carter Center	Inc D	40	16	0	0
Asclepias	*incarnata*	Carter Center	Inc D	41	16	0	0

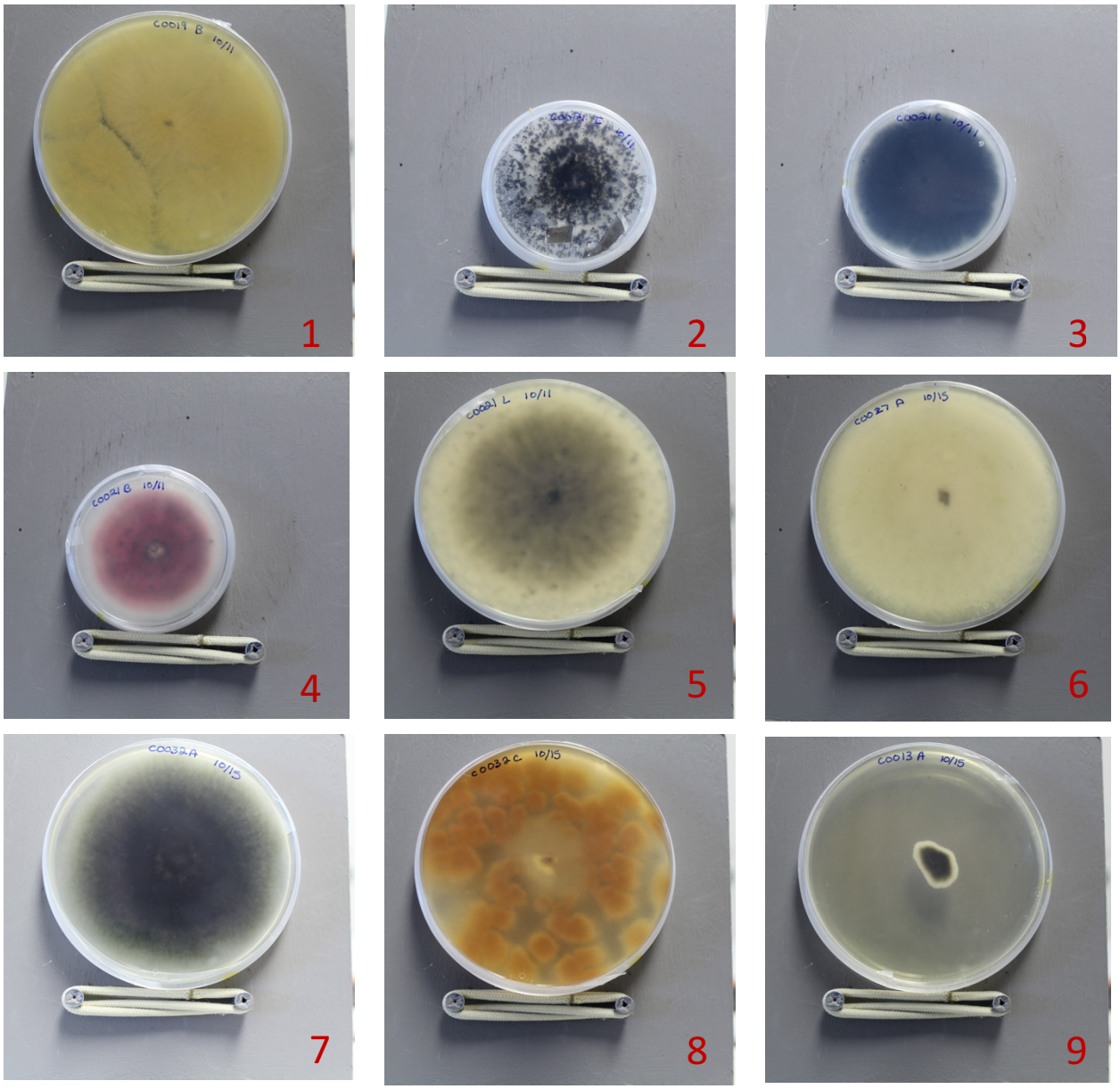
Asclepias	*incarnata*	Carter Center	Inc D	42	16	2	2
Asclepias	*incarnata*	Carter Center	Inc E	43	16	0	0
Asclepias	*incarnata*	Carter Center	Inc E	44	16	0	0
Asclepias	*incarnata*	Carter Center	Inc E	45	16	1	1
Asclepias	*incarnata*	De Roode Yard	Inc F	46	20	6	0
Asclepias	*incarnata*	De Roode Yard	Inc F	47	20	8	3
Asclepias	*incarnata*	De Roode Yard	Inc F	48	20	13	7
Asclepias	*incarnata*	De Roode Yard	Inc F	49	20	15	13
Asclepias	*incarnata*	Greenhouse	Inc G	50	20	2	2
Asclepias	*incarnata*	Greenhouse	Inc G	51	20	0	0
Asclepias	*incarnata*	Greenhouse	Inc G	52	20	0	0
Asclepias	*incarnata*	Greenhouse	Inc G	53	20	0	0
Asclepias	*incarnata*	Greenhouse	Inc G	54	20	0	0
Asclepias	*incarnata*	Greenhouse	Inc H	55	30	0	0
Asclepias	*incarnata*	Greenhouse	Inc H	56	25	2	2
Asclepias	*incarnata*	Greenhouse	Inc H	57	30	0	0
Asclepias	*incarnata*	Greenhouse	Inc H	58	30	0	0
Asclepias	*incarnata*	Greenhouse	Inc H	59	30	0	0
Asclepias	*incarnata*	Greenhouse	Inc I	60	30	3	3
Asclepias	*incarnata*	Greenhouse	Inc I	61	20	3	3
Asclepias	*incarnata*	Greenhouse	Inc I	62	27	3	2
Asclepias	*incarnata*	Greenhouse	Inc I	63	27	0	0
Asclepias	*incarnata*	Greenhouse	Inc I	64	30	0	0
Asclepias	*syriaca*	Fernbank	Syr A	68	7	7	7
Asclepias	*syriaca*	Fernbank	Syr A	69	9	7	7
Asclepias	*syriaca*	Fernbank	Syr A	70	7	7	7

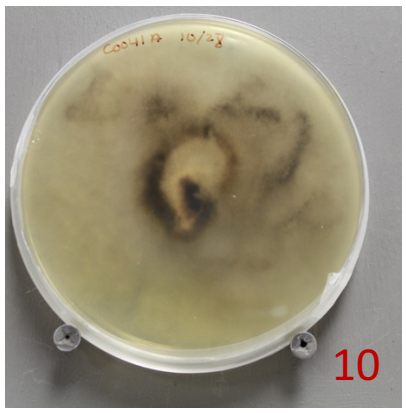
Asclepias	*syriaca*	Fernbank	Syr A	71	9	4	4
Asclepias	*syriaca*	Fernbank	Syr A	72	9	1	0
Asclepias	*syriaca*	Fernbank	Syr B	73	41	1	1
Asclepias	*syriaca*	Fernbank	Syr B	74	48	0	0
Asclepias	*syriaca*	Fernbank	Syr B	75	48	0	0
Asclepias	*tuberosa*	Fernbank	Tub A	76	13	13	13
Asclepias	*tuberosa*	Fernbank	Tub A	77	12	10	10
Asclepias	*tuberosa*	Fernbank	Tub A	78	16	16	16
Asclepias	*tuberosa*	Fernbank	Tub A	79	16	16	16
Asclepias	*tuberosa*	Fernbank	Tub A	80	16	16	16
Asclepias	*tuberosa*	Fernbank	Tub B	81	12	5	5
Asclepias	*tuberosa*	Fernbank	Tub B	82	10	5	5
Asclepias	*tuberosa*	Fernbank	Tub B	83	11	11	11
Asclepias	*tuberosa*	Fernbank	Tub B	84	8	8	8
Asclepias	*tuberosa*	Fernbank	Tub B	85	9	7	7
Asclepias	*tuberosa*	Fernbank	Tub C	86	40	7	3
Asclepias	*tuberosa*	Fernbank	Tub C	87	34	20	17
Asclepias	*tuberosa*	Fernbank	Tub C	88	38	17	13
Asclepias	*tuberosa*	Fernbank	Tub D	89	47	2	2
Asclepias	*tuberosa*	Fernbank	Tub D	90	41	3	3
Asclepias	*tuberosa*	Fernbank	Tub D	91	35	14	11
Asclepias	*tuberosa*	Fernbank	Tub E	92	37	10	5
Asclepias	*tuberosa*	Fernbank	Tub E	93	35	11	9
Asclepias	*tuberosa*	Fernbank	Tub E	94	41	1	1
Callicarpa	*sp.*	Gerardo Garden	Cal A	1	40	18	0
Callicarpa	*sp.*	Gerardo Garden	Cal A	2	24	5	0
Callicarpa	*sp.*	Gerardo Garden	Cal A	3	40	8	0
Rosa	*sp.*	Gerardo Garden	Ros A	65	40	0	0
Rosa	*sp.*	Gerardo Garden	Ros A	66	40	0	0

Rosa	*sp.*	Gerardo Garden	Ros A	67	40	0	0
Vaccinium	*sp.*	Gerardo Garden	Vac A	95	40	15	0
Vaccinium	*sp.*	Gerardo Garden	Vac A	96	40	24	0
Vaccinium	*sp.*	Gerardo Garden	Vac A	97	40	27	0

Appendix C

Fungal Endophyte Morphotype Group Classification Photos

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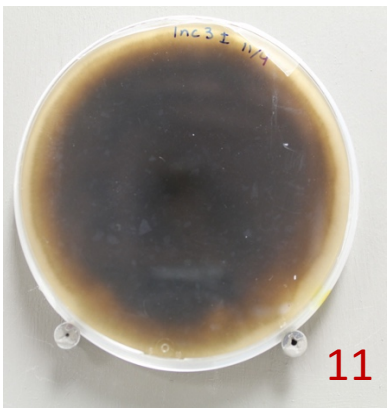
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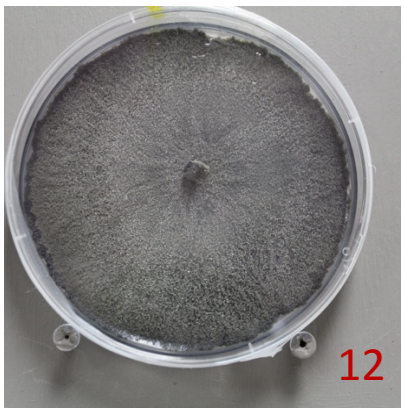
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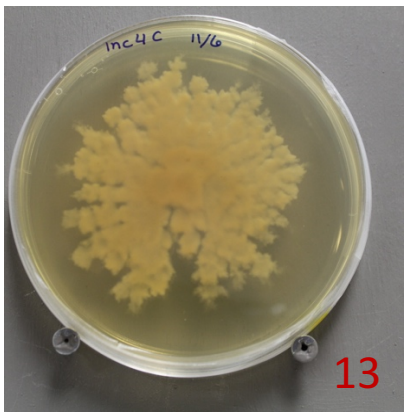
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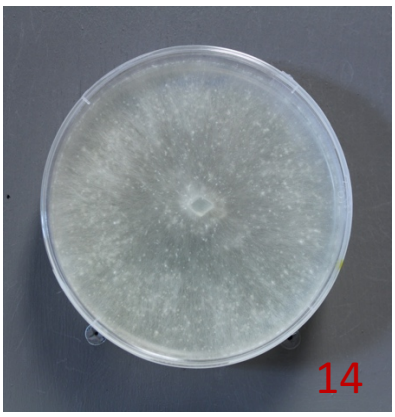
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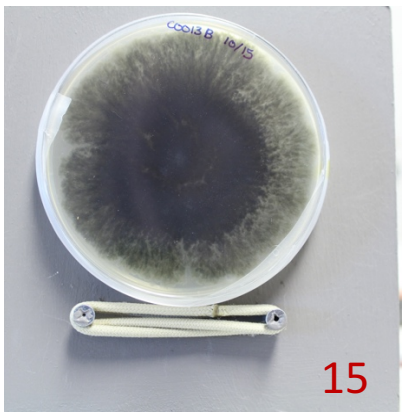
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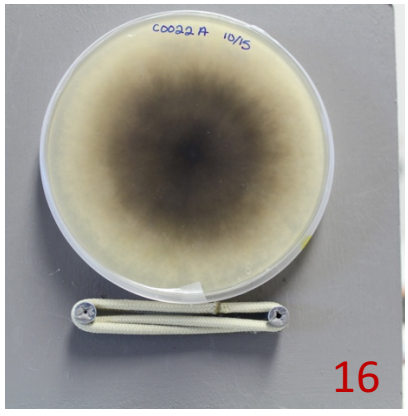
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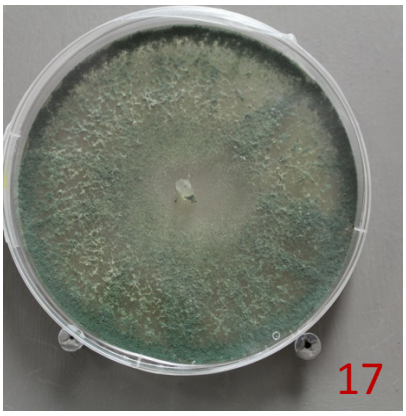
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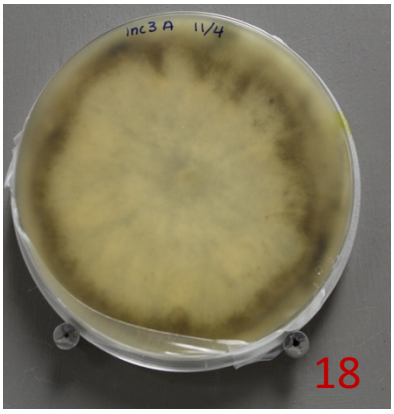
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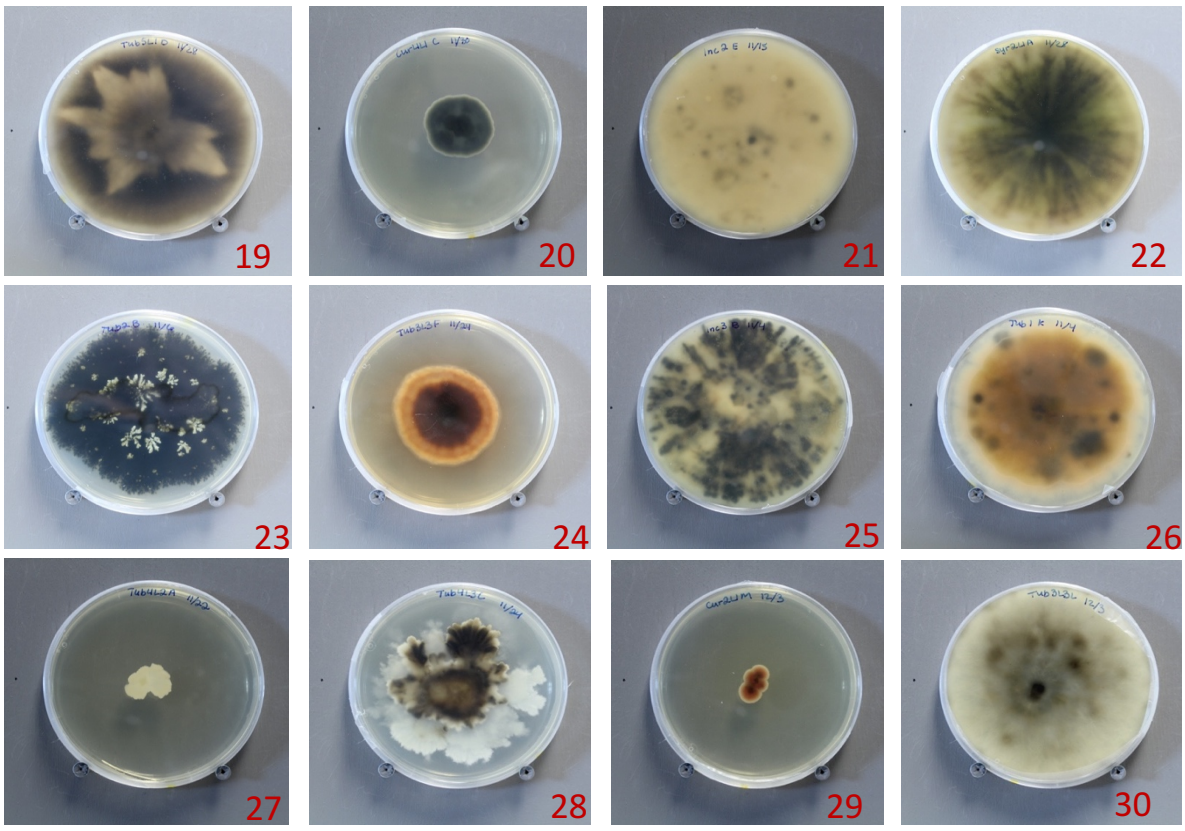
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