5-2017

Range-wide Prevalence and Impacts of Pseudocercosporella inconspicua on Lilium grayi and an Assessment of L. superbum and L. michauxii as Reservoirs

Cindy L. Barrett
East Tennessee State University

Range-wide Prevalence and Impacts of *Pseudocercosporella inconspicua* on *Lilium grayi* and an Assessment of *L. superbum* and *L. michauxii* as Reservoirs

---

A thesis

presented to

the faculty of the Department of Biology

East Tennessee State University

`

In partial fulfillment

of the requirements for the degree

Master of Science in Biology

---

by

Cindy Lynn Barrett

May 2017

Foster Levy, Ph.D., Chair

James T. Donaldson, M.S.

Aruna Kilaru, Ph.D.

Christopher Ulrey, Ph.D.

Keywords: *Pseudocercosporella inconspicua*, leaf spot disease, Gray's lily, Michaux's lily, Turk's Cap lily, Canada lily, *L. canadense*, disease inoculation, host

ABSTRACT

Range-wide Prevalence and Impacts of *Pseudocercosporella inconspicua* on *Lilium grayi* and an Assessment of *L. superbum* and *L. michauxii* as Reservoirs

by

Cindy Lynn Barrett

Lilium grayi (Gray's Lily), a southern Appalachian endemic species, is threatened by a *Lilium*-specific fungal pathogen, *Pseudocercosporella inconspicua*. The disease is characterized by tan lesions that can cause early senescence, while also lowering seed production and viability. This project tested for *P. inconspicua* conidia and accessed health at nine locations. The disease was present and ubiquitous across the range of *L. grayi*. Through identification of *P. inconspicua* conidia in the field, *L. superbum* (Turk's Cap Lily) was identified as an additional host, while *L. michauxii* (Michaux's Lily) was disease-free. However, infection was inducible in both species. With the disease widespread in *L. superbum* and this species represented by many large populations, *L. superbum* may act as disease reservoir, further complicating the outlook for *L. grayi*. The disease should be considered an epidemic because of its impact on individual plants, its commonness within populations, and its ubiquity across the geographical range.

ACKNOWLEDGEMENTS

For financial support: Friends of Roan Mountain and the Marcia Davis Research Award from East Tennessee State University.

Professor Donaldson, Dr. Levy, Dr. Walker for help with field work and critical analysis of this thesis.

Dr. Kilaru for use of laboratory.

For access and permission to conduct scientific research: Dr. Ulrey, the plant ecologist of the Blue Ridge Parkway; Marquette Crockett, the Southern Appalachian Highlands Conservancy Roan Stewardship Director; Tennessee State Parks; The Nature Conservancy; Tom Blevins of the George Washington and Jefferson National Forests.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Gray's Lily, *Lilium grayi* S. Watson, is a globally-rare lily that is restricted to a limited number of locations in the southeastern United States (Radford 1968; Weakley et al. 2012). *Lilium grayi* is a narrow, southern Appalachian endemic; its population structure varies greatly across its range from a small number of individuals (<10) to much larger occurrences (>100). The habitat is most commonly high elevation (>1000 m) seeps and grassy balds, but plants can also be found in boulder fields and lower elevation meadows, bogs, and wetlands (Radford 1968; Weakley et al. 2012).

During the last two decades, studies performed throughout the *L. grayi* range have noted a trend of declining, in-season health, early senescence, and failure to seed (Bates 1998; Donaldson 2003; Powell 2011; Ingram 2013; Ulrey pers. comm.). The pathogen responsible for these apparent disease symptoms was originally attributed to a species of *Colletotrichum* (Bates 1998). However, recent work has shown that *Colletotrichum* causes a secondary fungal infection, while the primary causal agent for lily leaf spot disease in *L. grayi* on Roan Mountain is the plant fungal pathogen, *Pseudocercosporella inconspicua* (G. Winter) U. Braun (Ingram 2013). This pathogen, a species in the division Ascomycota, is characterized by an ascus, a cup-shaped sporangium on which form the sexually derived ascospores (Trigiano 2008). Ascospores are resilient to degradation and are thought to form as a means to overwinter or when host resources are near exhaustion (Trigiano 2008).

Pseudocercosporella inconspicua can also produce spores asexually; these spores are called conidia. It is common in nature to observe conidia and the asexual life cycle in the absence of the sexual life cycle (Trigiano 2008). It is thought that conidia infect their hosts by

entering the leaf via stoma after which hyphae form and colonize intercellular spaces (Daub 2000). As the *P. inconspicua* infection progresses, it damages photosynthetic host tissue until visible lesions form. Damage to the host tissue may be caused by the release of cercosporin, a lipid-soluble toxin that is responsible for breaking down cell membranes to provide nutrition to the hyphae (Daub 2000).

Although the underlying disease pathology is unclear, the impacts of the pathogen on Roan Mountain are apparent. In a recent study, Ingram (2013) found that up to 94% of mature lilies had lily leaf spot disease by the end of the growing season. Stem mortality rates can reach 100% for non-reproducing lilies and up to 81% in reproductively mature lilies. While these mortality rates include death due to disease and other factors, disease was seen on all plants. The majority (68%) of reproductively mature lilies also had disease damage on developing seed capsules. The leaf spot disease may be especially damaging to *L. grayi* because in favorable conditions, *P. inconspicua* can undergo several asexual life cycles per season (Ingram 2013).

A 2017 study by Ingram et al. investigated the historic presence of *P. inconspicua* occurring on two lily species, *L. canadense* and *L. grayi*. Their study examined the herbarium records of more than 500 specimens represented in 6 major herbaria. *Pseudocercosporella inconspicua* lesions were present in only 3 of 526 herbarium sheets. The earliest specimen with infection in North America was from 1916, despite many earlier collections dating from the 1850's. In herbarium specimens, *P. inconspicua* appears regionally on Roan Mountain only as recently as 1947. These results led the researchers to posit that *P. inconspicua* is an introduced, exotic pathogen (Ingram et al. 2017). Their hypothesis is further supported by the knowledge that (i) *P. inconspicua* is host-specific to the genus *Lilium* and, (ii) of the eleven *Lilium* species listed as hosts, only four are found in North America (Braun 1995).

Introduced pathogens can have severe ecological and economic consequences on both a global and regional scale. Mack et al. (2000) state in a recent review that the ramifications of exotic introductions of all pathogen types cannot be underestimated and the "global cost of virulent plant and animal diseases caused by parasites transported to new ranges and presented with susceptible new hosts is currently incalculable." Of all plant pathogens, fungal infections are responsible for the majority of plant damage to both native species and cultivars (Fletcher et al. 2010).

There are several infamous examples of exotic fungal pathogens devastating host populations. For example, the chestnut blight caused by *Cryphonectria parasitica*, an exotic introduction to North America, was especially detrimental because it rendered American chestnuts, *Castanea dentata*, "functionally extinct" (Ellison et al. 2005). Loss of this foundation species has the potential to significantly alter forest ecology (Ellison et al. 2005). Another example of an exotic fungal pathogen with catastrophic impact is the recent global spread of the Asian soybean rust, *Phakopsora pachyrhizi*. This pathogen can be quite virulent and has the potential to decrease crop yields by up to 80% (Schnepf 2005). By traveling on wind currents from South Africa in 2001 to South America in 2002, the pathogen spread to three continents in only three years. It then spread to susceptible host species in the United States in 2004 (Fletcher et al. 2010). This rapid spread can be attributed, in part, to its broad host range of legume species (Fletcher et al. 2010).

If *P. inconspicua* is indeed an exotic introduction, this poses a particularly concerning threat given that, as a southern Appalachian endemic, the range of *L. grayi* is very limited. Moreover, any detrimental effects could easily cause local extinction in small populations of *L. grayi*. With potential damaging effects of exotic pathogens and the negative health impacts of *P.*

inconspicua of *L. grayi* on Roan Mountain, additional study of the geographic distribution of lily leaf spot disease and the host range of *P. inconspicua* was warranted. This research project extended the epidemiological work completed on Roan Mountain with two main goals.

The first goal was to assess the geographic occurrence and prevalence of *P. inconspicua* across the range of *L. grayi*. Disease occurrence and population prevalence were assessed by a nested, systematic approach. The first step was to determine if *P. inconspicua* occurs outside Roan Mountain. This was accomplished by inspection of suspected fungal lesion samples for *P. inconspicua* conidia taken from populations throughout the range of *L. grayi*. If *P. inconspicua* was present, demographic and health data were collected to determine the impacts of disease on plant health and reproduction. These data were also used to compare impacts among *L. grayi* populations. Environmental variables such as habitat type, canopy cover, and invertebrate browsing were investigated to determine whether their presence fostered an increased diseased state.

The second goal was to examine the host range of *P. inconspicua*, i.e. to determine if *P. inconspicua* infection occurs naturally on other regional lily species, *L. michauxii* and *L. superbum*. Each occurs within the range of *L. grayi*, but neither is a known host species of *P. inconspicua* (Braun 1988). First, populations of both species were examined and those with disease symptoms were tested for *P. inconspicua* conidia. Recovering *P. inconspicua* conidia from suspected fungal lesions would support the host species hypothesis. Second, experimental inoculations were used to test if *P. inconspicua* infection can be induced. Positive results in inoculation experiments would provide confirmation that either lily is a potential host species. In the field, *L. superbum* often exhibits symptoms similar to infected *L. grayi* plants. In contrast, only healthy *L. michauxii* plants have been observed by me and others. These observations led

to the question of whether *L. michauxii* is susceptible or resistant to the disease. To answer this question, *L. michauxii* were inoculated with *P. inconspicua*.

Experimental inoculations had three potential outcomes, each suggesting a separate mechanism to explain the apparent disease resistance of the healthy *L. michauxii* in natural populations. One, if no infection follows inoculation, this would suggest that *L. michauxii* is genetically resistant to the disease and that *L. michauxii* is not a host species. Two, if a limited or restrained infection develops after inoculation, this would suggest a hypersensitive response. Three, if extensive fungal infection follows inoculation, this would support characterization of *L. michauxii* as a potential host species but one that fails to contract disease in nature, perhaps due to mechanical exclusion of the disease conidia.

Thus, a prevalence study provides a glimpse into the range-wide disease burden of *L. grayi* as a species. If disease is prevalent and significantly reduces lily health throughout the *L. grayi* range, conservationists when reevaluating species viability should consider these impacts. Moreover, if disease is found on *L. superbum* or *L. michauxii*, this will extend the known host range of *P. inconspicua* and also identify potential disease reservoirs of *P. inconspicua*. Surveying populations of known hosts of *P. inconspicua*, such as *L. canadense*, would also further categorize disease reservoirs. Species deemed as disease reservoirs should be included in future disease management strategies of *L. grayi*.

CHAPTER 2

METHODS

Plant Identification

This study included four native lily species indigenous to the southern Appalachians: *L. canadense*, *L. grayi*, *L. michauxii*, and *L. superbum*. Height, elevation, and leaf morphology were not sufficient characters for identification, as the ranges and elevations overlapped and/or a character state was not unique to a species. For example, *L. superbum* individuals are on average the tallest of the four species studied. However, in shaded habitats *L. grayi*, *L. superbum*, and *L. canadense* can all be similar in height. Additionally, *Lilium grayi*, *L. superbum*, and *L. canadense* all have similar leaf morphology. For these species, identification relied on previously marked plots or on flower morphology, specifically the degree of recurve of the perianth. *Lilium superbum* and *L. michauxii* have nearly identical flower morphology, but leaf morphology differs. *Lilium michauxii* has an unmistakable oblanceolate leaf shape and a waxy, thicker cuticle than other locally-native *Lilium* species, and it is the only flower of these four species that is fragrant (Skinner 2002). Characteristics of the four species are summarized in Table 1.

Table 1. Characteristics of *Lilium* species included in the current study. Characters unique to each species are in bold and underlined.

	L. canadense	***L. grayi***	***L. michauxii***	***L. superbum***
Habitat	Wet meadows, bogs, and balds; in mountains	Balds, seeps, openings in mountains, boulder fields	**Drier habitats**; less commonly in bogs, upland woods, and thickets	Moist or wet meadows and coves; in mountains

Table 1. Continued.

		L. canadense	*L. grayi*	*L. michauxii*	*L. superbum*
Range		ne Canada and US; s US in mountains	Southern Appalachian endemic to sw VA, nw NC, and ne TN	Appalachian species	More widespread
Population size		Populations can be small (<10) or large (>100)	Populations can be small (<10) or large (>100)	Usually small populations (<10)	Often very large populations (>100)
Maximum height (meters)		2	2	0.4–1.3	3
Leaves	**Shape**	Elliptic to lanceolate	Elliptic to lanceolate	**Oblanceolate**	Elliptic – lanceolate
	Cuticle	Non-waxy	Non-waxy	**Waxy**	Non-waxy
	Size (cm)	4–13 x 0.8–2.5	4–13 x 0.8–2.5	3.6–11.1 x 1.5–3.8	8–18 x 1–3
	# of whorls	5–11	5–11	4–15	5–20
Flowers	**Color**	Orange to red to yellow	Red	Orange to reddish	Orange to reddish
	# of flowers	1–9	1–9	1–6	3–25
	Flowering time	June–early August	Late June–mid July	July–mid August	July-early August

Table 1. Continued.

		L. canadense	*L. grayi*	*L. michauxii*	*L. superbum*
Flowers	**Major pollinator**	Ruby-throated hummingbird	Ruby-throated hummingbirds	Swallowtail butterflies	Swallowtail butterflies
	Fragrance	None	None	**Fragrant**	None
	Degree of perianth recurve	**Slightly recurved or spreading**. 	**Slight to none** 	Strongly reflexed 	Strongly reflexed 

Sample Sites

To assess prevalence of *Pseudocercosporella inconspicua* throughout the range of *L. grayi*, nine locations in three states were selected and in tandem, represent the extent of the *L. grayi* geographic range. The Cloudland Hotel Site (Carter County and Mitchell County), the Rhododendron Gardens (Mitchell County), and Big Yellow Mountain (Avery County) all of which belong to the Roan Mountain massif were surveyed. Outside the Roan Mountain massif, samples were taken from Bluff Mountain (Ashe County) in North Carolina. In Virginia, Whitetop Mountain (Grayson and Washington County) was included in the study. Four

additional subpopulations of a population complex, also in North Carolina, were surveyed along the Blue Ridge Parkway. Specific sites of the latter area cannot be published due to the threatened status of the species (Finnegan 2014) and concerns for vandalism. In fact, most *L. grayi* locations in this study are protected by state, federal, or non-governmental agencies, so only approximate geographic coordinates are provided. Interested parties must contact the agencies that manage these areas for precise locations, access, and approval to conduct scientific research.

Determining *P. inconspicua* Presence on Native Lily Species

Observations of *L. michauxii* were made across the region (Table 2). *Lilium superbum* lesion samples were tested for *P. inconspicua* at three sites: Unaka Mountain (Unicoi County), Holston Mountain (Sullivan County), and Roan Mountain (Carter County), all in Tennessee (Table 3). *Lilium canadense* observations were made at Shady Valley (Table 3; Figure 1).

Table 2. Observations of *L. michauxii*, all of which were disease-free.

Location	Date	County, State	Number of flowering individuals	Number of nonflowering individuals	Disease symptoms	Observer*
Unaka Mt.	25May2015	Unicoi, TN	0	1	No	cb, fl
Route 19E, Appalachian Trail	19Jun2015	Carter, TN	2	7	No	fl, ew
Holston Mt., Trail 22	3Jun2015	Sullivan, TN	0	1	No	cb, fl
Stone Mt.	29Jun2015	Carter, TN	5	5	No	fl, ew
Holston Mt., Flint	29Jun2015	Sullivan, TN	4	0	No	ms

Table 2. Continued.

Location	Date	County, State	Number of flowering individuals	Number of nonflowering individuals	Disease symptoms	Observer*
Pinnacle Trail	5Jul2015	Unicoi, TN	3	2	No	fl, ew
Little Mt.	7Jul2015	Unicoi, TN	1	1	No	fl, ew
Rocky Fork, Xerophyllum Bald	15Jul2015	Greene, TN	4	0	No	fl, ew
Rocky Fork, Pine Ridge	15Jul2015	Unicoi, TN	2	0	No**	fl, ew
Buck Mt. Road	29Jul2015	Avery, NC	1	0	No	fl, ew
Indian Grave Gap, Appalachian Trail South	1Aug2015	Unicoi, TN	1	0	No	fl, ew
Scioto Road	2Aug2015	Unicoi, TN	7	0	No**	fl, ew
Bluff Mt.	4Aug2015	Ashe, NC	1	0	No	cb
Round Knob Mt.	9June2015	Greene, TN	0	1	No	fl, ew, am
Totals: 15 sites	**4 months**	**6 counties 2 NC/ 4 TN**	**32 flowering**	**18 nonflowering**	**0 disease symptoms**	**5 observers**

*Observer Key: am=Adam McCullough; cb=Cindy Barrett; ew=Elaine Walker; fl=Foster Levy; ms=Martin Silver.
** Lesion was sampled as a precaution, but no *P. inconspicua* conidia were present.

Table 3. Populations of *L. grayi* and *L. superbum* surveyed in the 2015 season.

Sites	Sub-populations	Species surveyed	Date of 1st demographic survey	Date of 2nd demographic survey	County, state	Approximate geographic coordinates
Blue Ridge Parkway	4	*L. grayi*	15Jun2015		Confidential	Confidential
Roan Mt.	2	*L. grayi*	22Jun2015	3Aug2015	Mitchell, NC; Carter, TN	36.1042800ºN -82.084500ºW
Big Yellow Mt.	2	*L. grayi*	29Jun2015	14Aug2015	Avery, NC	36.1101200°N -82.027000°W
Grassy Ridge	1	*L. grayi*	29Jun2015	**	Avery, NC	36.1042800ºN -82.084500ºW
Little Hump Mt. Big Hump Mt.	1	N/A	**	**	Avery, NC	36.1384500ºN -81.990300ºW
Whitetop Mt.	5	*L. grayi* *	9Jul2015	17Aug2015	Grayson, VA; Washington, VA	36.6387200ºN -81.605300ºW
Bluff Mt.	2	*	*	*	Ashe, NC	35.841200ºN -82.906500ºW
Roan Mt. State Park	3	*L. superbum*	20May2015	7Sep2015	Carter, TN	36.1042800ºN -82.084500ºW
Unaka Mt.	3	*L. superbum*	25May2015	***	Unicoi, TN	36.1334400ºN -82.2965200W
Holston Mt.	3	*L. superbum*	3Jun2015	***	Carter, TN	36.457600ºN -82.077300ºW

*Several attempts were made to locate an inflorescence or infructescence of *L. grayi* at Whitetop Mountain (25Jun2015; 9Jul2015; and 31Jul2015) and Bluff Mountain (7Jul2015; 1Aug2015; and 5Aug2015). The emerging individuals at both Whitetop and Bluff Mountains were discovered to be *L. superbum* lilies not *L. grayi*. Other data gathered from Whitetop Mountain was from known *L. grayi* individuals.
**Three attempts were made to locate *L. grayi* lilies at Little Hump Mountain (30Jun2015; 13Jul2015; 14Jul2015), but access was unattainable because of the strenuous nature of the trail and inclement weather that made the trail dangerous. Inclement weather (13Jul2015 and 14Jul2015) prevented the second demographic data collection at the Grassy Ridge site.
***Focus was placed on *L. grayi* monitoring because depletion of funds prevented a second demographic census trip to these *L. superbum* sites.

Figure 1. Healthy *L. canadense* at Shady Valley, TN. Photograph on left was taken on July 1, 2015 and the photograph on right was taken August 9, 2015.

Aseptic Protocol

To minimize the chance that the researchers would become vectors for fungal spore transmission, the following aseptic protocols were followed: only one site was visited per day, clean clothes were worn to each site, shoes were sanitized with a 10% aqueous solution of bleach or washed between trips, latex gloves were worn while handling plants, and tools were disinfected with a 10% bleach solution between interplant contacts.

Site Characteristics and Plant Demographic Data

Demographic data was collected for each population. Because Ingram (2013) found a correlation between disease and proximity of individuals to trail transects on Roan Mountain, only *Lilium* plants that were one meter or farther away from transects were included in the present study. Two site characteristics were recorded for each population, type of habitat (open bald, forest, seep, bog), and elevation. Invertebrate herbivory was noted. If possible, twenty-five plants were censused per subpopulation and three subpopulations were surveyed per site.

To be considered a different subpopulation, plant clusters were at least 25 meters or farther away from other lily clusters. Plant height, number of leaf whorls, number of flowers and capsules, and a health scale score were recorded for each plant. A visual health assessment was scored on a ten-point scale based on a rating of one to five with half point increments. A healthy plant was scored as a 5, a score of 2.5 corresponded to a plant with 50% of the total plant area with symptoms of disease, and a dead plant received a score of 1. The remainder of the scale corresponded to similar incremental differences. To measure the effects of the fungal pathogen on in-season health, each population was re-censused near the end of the growing season, i.e. usually in September. All sites were visited twice during the 2015 growing season (Table 3), between late May and September. Select sites were visited in the 2016 growing season (Table 4).

Table 4. Populations of *L. canadense*, *L. grayi*, and *L. superbum* surveyed in the 2016 season.

Site	**Sub-populations**	**Species surveyed**	**Date of 1st demographic Survey**	**Date of 2nd demographic survey**	**County, state**	**Approximate geographic coordinates**
Whitetop Mt.	1	*L. grayi*	31May2016	11Sep2016	Grayson, VA; Washington, VA	36.6387200ºN -81.605300ºW
BRP1	1	*L. grayi*	21Jun2016	10Sep2016	Confidential	Confidential
Roan Mt.	1	*L. grayi*	30Jun2016	2Sep2016	Mitchell, NC; Carter, TN	36.1042800ºN -82.084500ºW
Elk Hollow Preserve	1	*L. grayi*	28Jun2016	18Jul2016	Avery, NC	36.063700ºN -82.012600ºW
Shady Valley	1	*L. canadense*	1Jul2016	9Aug2016	Johnson, TN	36.519300ºN -81.927900ºW
Roan Mt. State Park	3	*L. superbum*	8June2016	8Sep2016	Carter, TN	36.1042800ºN -82.084500ºW

Conidia Collection

Pseudocercosporella inconspicua infection manifests as characteristic elliptical, tan lesions approximately 3–20 mm in diameter, which can elongate to up to 4 cm in length. The lesions exhibit a slightly textured gray center encircled by a brown halo (Braun 1988). As the season progressed, the health of the entire plant was detrimentally affected, showing chlorotic leaves that lead to tissue wilt and withering, often leading to early senescence of all the aboveground structures of the plant (Ingram 2013).

A visual diagnosis of the fungal lesion was made prior to sample collection. Samples were examined for visual evidence of *P. inconspicua* conidia. This process was non-invasive to the lily plants. In the field, a leaf containing a fungal lesion matching the above description was touched to a microscope slide. A small drop of acid fuchsin stain was applied to the microscope slide followed by a cover slip. To preserve the slide, clear nail polish was applied around the cover slip. Four methods of collecting conidia were evaluated (touch sampling, wet mount, tape collection, and staining the conidia sample) before deciding that staining the conidia with acid fuchsin was the preferred method. This sampling method incorporates the advantages of all other collection techniques because; (i) the liquid stain disperses the spores from the initial touch sample, (ii) the stain is toxic to *P. inconspicua* thereby preventing germination of the fungal conidia (Ingram pers. comm.), (iii) the stain colors the hyaline conidia allowing for easier viewing, discovery, and measurement, and (iv) sealing the slide prevents distortion of the conidia through desiccation.

In the 2015 season, *L. grayi* and *L. superbum* lesions were inspected for *P. inconspicua* conidia. In 2016, sampling was expanded to include *L. canadense*. No *L. michauxii* were examined for conidia because all 50 *L. michauxii* individuals surveyed in both 2015 and 2016

appeared healthy and disease-free, so there were no characteristic *P. inconspicua* fungal lesions to sample (Table 2; Figure 2). One minimally suspect lesion was sampled as a precaution, but no *P. inconspicua* conidia were present (Table 2).

Figure 2. Late season photographs taken of a senesced *L. grayi* (left) and healthy *L. michauxii* (right) on Bluff Mountain on August 4, 2015.

Conidia Identification

In the laboratory, a definitive diagnosis of *P. inconspicua* was accomplished through identification of characteristic, microscopic, conidial morphology as described by Braun (1988). The conidia are smooth and hyaline, 1–7 septate and slender, measuring 30–110 µm in length and 2–6 µm in width. The base of each conidium appears blunt or truncated, and the apex is tapered. A conidial scar is also present where the spore was released from the conidiophore, but the scar is not thickened as in related species. The conidiophore grows internally through the leaf and emerges through the cuticle. The conidiophores are arranged singly or in loose groups; they are smooth, aseptate, and measure 5–25 x 2.5–8 µm (Braun 1998).

Fungal Inoculations: Experimental Design and Inoculum

To experimentally examine the host range of *P. inconspicua*, two regional lily species, *L. michauxii* and *L. superbum*, were inoculated with *P. inconspicua* in the laboratory. *Lilium grayi* served as a control in this experiment because it is a known host for *P. inconspicua* and similar inoculation trials have proven successful (Ingram 2013). While *L. superbum* showed disease symptoms in the field, plants of this species were inoculated because a positive result would provide confirmation that *L. superbum* is a host species for *P. inconspicua*. *Lilium michauxii* was included in the inoculation experiments, as its geographical range overlaps that of *L. canadense, L. gray,* and *L. superbum*. Most importantly, however, *L. michauxii* was included because it presents disease-free in the field (Figure 2). Inoculation experiments were included to determine whether this species has the potential to act as a host for *P. inconspicua*. If found to be a host, then inoculations can help elucidate the underlying mechanism of the observed absence of disease in the field. A positive result in the inoculation experiment would suggest mechanical exclusion, by the thick cuticle. A negative result would point to genetic resistance.

Lesion development was assessed in each fungal inoculation experiment to determine if varying degrees of susceptibility were present among the three lily species. In addition to this, two inoculum sources were utilized to determine if inoculum from *L. grayi* acted differentially to *P. inconspicua* inoculum from *L. superbum* on the three native lily species. Varying degrees of virulence or differing disease pathology among inoculum from differing hosts would suggest putative species-specific strains of *P. inconspicua*. Consequently, two sources of inoculum were used. The first was from a pure hyphal *P. inconspicua* culture derived from a collection on an individual of *L. grayi* on Roan Mountain, kindly provided by Russell Ingram. The second was *P. inconspicua* conidial lesions gathered from *L. superbum* individuals in the field. This inoculum

choice was made because pure hyphal culture of *P. inconspicua* from *L. superbum* was not available, as isolation of the *P. inconspicua* samples to pure culture was not possible while the test plants were still viable. It is acknowledged that inoculum type and source host species are conflated in this design.

Before lesions were used as inoculum, the presence of conidia and their identification as *P. inconspicua* was confirmed through visualization of microscopic conidial morphology as previously described. To confirm identity and yet preserve enough conidia for inoculum, each lesion was held above a microscope slide and the lesions were lightly scraped with a pointed, sterile razor blade. Only lesions which produced positive identification of *P. inconspicua* were used as inoculum. Lesions that did not yield *P. inconspicua* conidia or produced slight amounts of conidia were discarded.

Host Plant Collection

To acquire plant material for inoculation trials, bulbs of each species were gathered in the fall of 2015. Appropriate permits were secured from the appropriate agencies. Three *L. grayi bulbs* were gathered on 11Nov2015 at Roan Mountain. Three bulbs of *L. superbum* and one *L. michauxii* bulb were collected on 21Oct2015 in the Cherokee National Forest.

All plants were transferred to new potting soil to minimize exposure to conidia residing in native soil. Pine bark mulch was added to the bottom of the pots for drainage. All plants and bulbs were kept at 4 ˚C from the time of collection to simulate winter dormancy. After five months, the plants were grown at room temperature until leaves expanded. Approximately four weeks were needed to grow full-sized plants from bulbs.

For adequate sample size of each fungal inoculation experiment, the following additional plants were collected in the spring of 2016: 5 *L. grayi*, 3 *L. michauxii*, and 12 *L. superbum*.

Appropriate permits were secured or expressed permission was obtained to collect these plant specimens from the Cherokee and Pisgah National Forests.

Inoculation Protocol

In the first inoculation experiment *L. grayi*, *L. michauxii*, and *L. superbum* leaves were abraded and inoculated with pure *P. inconspicua* hyphal culture derived from *L. grayi*. On each plant, two leaves were inoculated with two abrasions per leaf (Figure 3). Abrasions were made with either sterile lancets or sterile razor blades as the smallest cork borer was too wide for the narrow *Lilium* leaves. A small piece of inoculum comprised of hyphae on potato dextrose agar (PDA), approximately 3 mm x 3 mm, was excised and placed on the abraded area. There were no observable conidia in the inocula. There were two control leaves per plant with two abrasions per leaf. On the control leaves, a small amount of sterile PDA media, approximately 3 mm x 3 mm, was placed on the abrasions. Plants in the first inoculation experiment were placed at room temperature in a sunny windowsill. The *Lilium* species included in this study require high light and cool temperatures, so after wilting was observed on the windowsill, the plants were removed to a more sheltered location.

Figure 3. Experimental *L. superbum* plant in fungal inoculation trial three depicting the two experimental leaves with two inoculated abrasions and two controls leaves that were not inoculated. This experimental setup was used on all experimental plants in the fungal inoculation trials.

The second inoculation experiment was identical to the first except plants were placed in a growth chamber after inoculation. This allowed the plants to receive adequate light without intense heat. The growth chamber was set for 12 hours of light at 15 ºC followed by 12 hours of darkness at 10 ºC. Test plants were monitored every two to three days for fungal lesion development.

The third inoculation experiment used *P. inconspicua* conidial inoculum from *L. superbum* individuals to inoculate *L. grayi, L. michauxii,* and *L. superbum* species. After the test plant leaves were abraded, a leaf lesion was touched to the injury. Two control leaves were included per plant with two abrasions per leaf. One control leaf was abraded and an asymptomatic *L. superbum* leaf was touched to the injury. The other control leaf was only

abraded. Leaves were abraded in the same manner and number as the previous inoculation experiments.

Unless noted otherwise (Table 5), intact plants were used in the experiment. In each experiment, lesion size was recorded at the longest and widest points and after symptoms appeared, photographs were taken to document plant health, degree of chlorosis, and fungal lesion progression. The information for all inoculation experiments is summarized in Table 5.

Table 5. Fungal Inoculation Experiment Summary. Reproductive maturity defined as greater than four leaf whorls.

Experiment	**Inoculum type**	**Inoculum source**	**Plants inoculated**	**Reproductive maturity**	**Experimental leaves / abrasions per leaf**	**Control leaves / abrasions per leaf**	**Conditions after inoculation**
Fungal Ex. 1	Hyphae	Pure culture from *L. grayi*	2 *L. superbum* 1 *L. michauxii* (cut stem)	1 *L. superbum*	2/2	2/2	Indoor Conditions*
Fungal Ex. 2	Hyphae	Pure culture from *L. grayi*	2 *L. grayi* (one cut stem) 1 *L. michauxii* 2 *L. superbum*	1 *L. superbum*	2/2	2/2	Growth Chamber;*** 1 mature *L. superbum* at Indoor Conditions*
Fungal Ex. 3	Conidia	Field collected from *L. superum*	1 *L. grayi* 1 *L. michauxii* 2 *L. superbum*	1 *L. grayi* 1 *L. michauxii* 1 *L. superbum*	2/2	2/2	Growth Chamber***
Field Ex. 1	Conidia	Field collected from *L. superum*	6 *L. superbum*	3 *L. superbum*	2/1	2/1	Outdoor Conditions**

*Indoor conditions: approximately 14 hours day and 10 hours night at approximately 21 ºC.
**Outdoor conditions: approximately 14 hours day and 10 hours night at ambient temperatures.
***Growth chamber: 12 hours light at 15 ºC and 12 hours dark at 10 ºC.

Field Inoculation Experiment

Field inoculation experiments were conducted to further clarify the host range of *P. inconspicua*. Field inoculation has two benefits over laboratory experimentation. One, the host plants were not disturbed and experienced no transplant shock. Two, field experiments were carried out under environmental conditions (humidity, rain, UVB light, and fluctuating temperatures, etc.) the pathogen naturally experiences.

A field inoculation trial was conducted at the Unaka Mountain site. Due to concerns for releasing a non-native, phytopathogen genotype into the field, the pure *P. inconspicua* culture (from *L. grayi*) was not used. Instead, local inoculum was collected. *Lilium michauxii* individuals were excluded from this component of the study. Although *L. michauxii* individuals appear unaffected in the field (even in the apparent presence of *P. inconspicua* conidia), a conservative approach was followed due to concerns for infecting a naïve plant species. Moreover, since similar experiments were already conducted on *L. grayi* (Ingram 2013), only *L. superbum* individuals were included in field experimentation. For inoculum, several infected *L. superbum* leaves bearing characteristic lesions were collected. Healthy *L. superbum* plants were identified and two experimental and two control leaves were abraded. A lesion matching the above description was touched to the abrasion on the experimental leaves. The control leaves were only abraded. If possible, leaves on upper whorls were chosen, as this would limit potential contamination via rain-splash from spores in the leaf litter. A sample of the inoculum used was taken to the laboratory to confirm the fungal lesion was infected with *P. inconspicua*. The lab inspections confirmed that each inoculum sample was infected with *P. inconspicua*.

Unfortunately, data integrity could not be maintained due to human vandalism and the first field experiment conducted at Unaka Mountain on 20Jun2016 was abandoned.

It should be noted for future researchers that when lesions on *L. grayi* and *L. superbum* do not test positive for the presence of *P. inconspicua* conidia, it is premature to conclude these lesions were caused by another pathogen because light rain can wash the adaxial and abaxial lesion surfaces completely clean of conidia. Conidia may not reform for several days. For example, sampling on 2June2016, 13Jun2016, and 14Jun2016 in Roan Mountain State Park yielded no conidia in a previously positive area. Without the mass of conidia, the *P. inconspicua* lesions also appear darker.

Pure Culture Isolation

Attempts to obtain a pure *P. inconspicua* culture from *L. superbum* were successful, however pure culture was gained after the inoculation experiments had concluded. For future researchers a description of the successful protocol follows. A lesion bearing conidia was touched to a prepared plate of PDA media and a three phase plate streak was immediately preformed, as done in bacteria isolation (Hertsenberg & Noori 2010). To obtain a pure culture this way, one must anticipate which colonies are *P. inconspicua*. *Pseudocercosporella inconspicua* grows slowly in culture; so, after three days, the slowest growing colonies were transferred to fresh media plates. Waiting longer allowed the more aggressive, secondary pathogens to overtake and occlude the *P. inconspicua* colonies. If fungal growth was seen the day after plating or if colonies were large after three days, these colonies were discarded. Promising cultures were continued at 20 ºC–24 ºC and monitored for 8–12 weeks after plating. In six days, the colonies have grown to their full size and were a light tan color; later, the colonies of *P. inconspicua* turned black and developed a white fluffy center. All cultures, whether isolated from leaf samples or conidial streak, were maintained until colony color developed. Colonies with a color that was not tan or black were discarded.

Data Analysis: Demographic Data

IBM SPSS version 23 was used to analyze demographic data. First, data were viewed by the Descriptives procedure to corroborate sample size and review for input errors. The Descriptives procedure was also used to generate the descriptive statistics including the mean and variance for height, health, and number of whorls for each species at each site.

A correlation analysis was performed to test for an association between the variables height, whorls, habitat, and health in *L. grayi* and *L. superbum* using the Bivariate Correlation procedure.

To determine if the health of *L. grayi* differed significantly among several explanatory variables (including: population, plant height, elevation, habitat, light condition, and browsing), a series of Kruskal-Wallis tests were performed. To determine if the health of *L. grayi* differed significantly between survey years a Kruskal-Wallis test was performed. To access effects of disease on reproduction, a Kruskal-Wallis test was utilized to determine if a significant difference was found between the number of flowers in the first survey and the number of capsules in the second survey. A Kruskal-Wallis test is the non-parametric version of the one-way ANOVA, and is the most appropriate test to utilize, as the dependent variable, health and flowers, was measured on an ordinal scale and the distribution of health was not normally distributed among the variables (Lund 2015). The same statistical analyses mentioned above were used to analyze data from *L. superbum*.

Data Analysis: Fungal Inoculation Experiments Lesion and Area of Chlorotic Zone

To determine if a difference in lesion size area was seen through time, among the three species, and between inoculum over the course of approximately 30 days post infection with *P. inconspicua*, a two-way repeated measures ANOVA was conducted. Outliers were identified

through boxplot visualization. The Shapiro-Wilk test was used to test the assumption of normality. The assumption of sphericity was tested using the Mauchly's sphericity test. If the sphericity assumption was violated, a Greenhouse-Geisser correction was used (Lund 2015). Area of chlorotic zone was analyzed in a similar manner.

Data Analysis: Lesion Development after Inoculation

A 2x2 Fisher's exact test was utilized to determine if the abrasion had an equal chance of developing a lesion between each inoculum type used—*P. inconspicua* hyphae from *L. grayi* or *P. inconspicua* conidia from *L. superbum*. A 2x3 Fischer's exact test was utilized to determine if the abrasion had an equal chance of developing a lesion between each species inoculated—*L. grayi*, *L. michauxii*, and *L. superbum*.

Data Analysis: Incubation Period, Days until Leaf Senescence, and Plant Senescence

To determine if there was a difference in incubation period, days until leaf senescence, or days until plant senescence, each measurement was analyzed separately with a two-way ANOVA. Boxplot visualization was used to identify outliers. Normality was assessed using the Shapiro-Wilk's test. The equality of variance was assessed using the Levene's test (Lund 2015).

CHAPTER 3

RESULTS

Demography and Health

Demography and Health of *L. grayi*

In 2015, nine *L. grayi* populations were monitored. Sample sites included: Bluff Mountain, Roan Mountain massif (Big Yellow Mountain, Rhododendron Gardens, Cloudland Hotel, and Grassy Ridge), Whitetop Mountain, and four sites along the Blue Ridge Parkway) with a total of 148 total individuals censused among the populations at one to three subpopulations per population (Table 3). *Pseudocercosporella inconspicua* conidia was identified at all the *L. grayi* locations sampled (Table 3; Table 4; Figure 4). The average *L. grayi* had three leaf whorls, was 64.5 cm tall, and had a health scale score of 2.9 (Table 6). The majority of plants did not flower (Table 6). The Grassy Ridge population had *L. grayi* individuals with the highest health score ($\bar{x}$ = 4.8). Other populations with high health scores were Roan Mountain ($\bar{x}$ = 4.1) and Big Yellow Mountain ($\bar{x}$ = 4.0). Whitetop Mountain had the lowest mean health score ($\bar{x}$ = 1.4). The Grassy Ridge and Big Yellow Mountain populations had the tallest plants ($\bar{x}$ = 89.0 cm and $\bar{x}$ = 88 cm, respectively), while Whitetop Mountain had the shortest plants ($\bar{x}$ = 37.2 cm). Table 7 shows a summary of data for height, number of whorls, and health score by population for *L. grayi*.

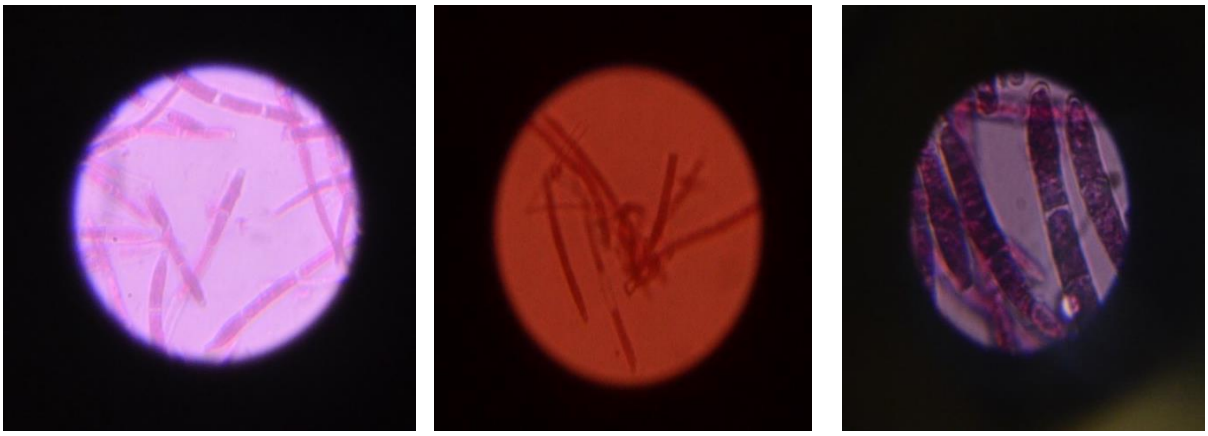


Figure 4. Examples of *P. inconspicua* conidia taken from across the range of *L. grayi* in 2015. Photographs were taken at varying levels of magnification. Hyaline conidia appear colored, as acid fuchsin stain was used to aid in conidia visualization.

Table 6: Summary descriptive statistics summary for height, number of whorls, and health score of *L. grayi* and *L. superbum* in 2015. N = 148 for *L. grayi*; N = 225 *L. superbum*.

	Species	Range	Mean	Standard Deviation
Height (cm)	*L. grayi*	10–130	64.5	26.7
	L. superbum	7–139	14.8	29.9
Number of Whorls	*L. grayi*	1–7	3.7	1.4
	L. superbum	1–10	2.8	2.2
Health Score	*L. grayi*	1–5	2.9	1.4
	L. superbum	1–5	3.2	1.5
Flowers	*L. grayi*	0–4	0.5	0.6

Table 7. *Lilium grayi* population summary of health score, height, and number of whorls. Standard deviation represented as s.d. and sample size as N.

Population	Sub-population Number	N	Health Score (1.0–5.0)			Height (cm)			Number of Whorls		
			Mean	Range	s.d.	Mean	Range	s.d.	Mean	Range	s.d.
Big Yellow Mt.	1	1	4.0	4.0–4.0	0.0	88.0	88–88	0.0	5.0	5–5	0.0
Blue Ridge Parkway 1	1	25	2.5	1.0–4.5	1.3	56.1	27–120	27.1	3.4	2–5	0.9
Blue Ridge Parkway 2	1	24	2.0	1.0–4.5	1.4	57.3	17–109	27.3	3.1	1–6	1.3
Blue Ridge Parkway 3	1	27	2.8	1.0–5.0	1.6	60.0	10–111	25.1	3.2	1–6	1.0
Blue Ridge Parkway 4	1	44	2.9	1.0–5.0	1.2	72.1	18–130	27.5	4.0	2–6	1.2
Roan Mt.	3	18	4.1	2.5–5.0	0.8	72.0	39–103	14.5	4.9	3–7	1.2
Grassy Ridge	1	5	4.8	4.5–5.0	0.3	89.0	58–119	22.6	6.0	4–7	1.4
Whitetop Mt.	1	4	1.5	1.0–2.0	0.6	37.2	18–58	21.7	2.2	1–4	1.5

Demography and Health of *L. superbum*

In 2015, three *L. superbum* populations were monitored (Roan Mountain State Park, Unaka Mountain, and Holston Mountain) with a total of 228 individuals censused among the three populations with three subpopulations per population. Each population had approximately the same number of observations (n ~75) with each subpopulation having approximately 25 observations. The average *L. superbum* had three leaf whorls, was 41.8 cm tall, and had a health scale score of 3.2 (Table 6). Holston Mountain had individuals with the highest health score ($\bar{x}$ = 3.8) and the tallest plants ($\bar{x}$ = 53.0 cm). Roan Mountain State Park had the lowest mean health

($\bar{x}$ = 2.8). The shortest plants on average were from the Unaka population ($\bar{x}$ = 32.7 cm). Table 8 shows summary data for health, height, and number of whorls by population for *L. superbum*.

Table 8. *Lilium superbum* population summary of health score, height, and number of whorls in 2015. Standard deviation represented as s.d. and sample size as N.

Population	**Sub-population Number**	**N**	**Health Score (1.0–5.0)**			**Height (cm)**			**Number of Whorls**		
			Mean	Range	s.d.	Mean	Range	s.d.	Mean	Range	s.d.
Holston Mt.	3	76	3.8	1.0–5.0	1.3	52.6	7–139	35.3	3.7	1–10	2.4
Roan Mt. State Park	3	75	2.8	1.0–5.0	1.5	40.0	10–115	21.9	2.7	1–9	1.8
Unaka Mt.	3	74	3.0	1.0–5.0	1.5	32.7	10–138	27.7	2.0	1–9	1.8

Variables Correlated with Health in *L. grayi* and *L. superbum*

For *L. grayi*, height, number of whorls, elevation, habitat, and light condition were all correlated with health. A similar result was found for *L. superbum*, with the exception that habitat was not correlated with health. Only *L. superbum* growing in forests were censused, creating a uniform variable that could not be analyzed. Table 9 and 10 show correlation summaries for *L. grayi* and *L. superbum*, respectively.

Table 9. Correlations of *L. grayi* for height, number of whorls, health, elevation, habitat, and light conditions. Bold indicates a correlation that was significant at the 0.01 level (2-tailed). Pearson's correlation coefficient (two-tailed) represented by R. N = 148 for *L. grayi*. Bold indicates a significant correlation, where $p < 0.05$.

		Height	Number of Whorls	Health	Elevation	Habitat	Light Conditions
Height	R	-	**0.76**	**0.59**	0.04	0.10	-0.12
	p-value	-	**<0.001**	**<0.001**	0.632	0.239	0.15
Number of Whorls	R	**0.76**	-	**0.60**	**0.27**	**0.34**	**-0.34**
	p-value	**<0.001**	-	**<0.001**	**0.001**	**<0.001**	**<0.001**
Health	R	**0.59**	**0.60**	-	**0.24**	**0.32**	**-0.32**
	p-value	**<0.001**	**<0.001**	-	**0.004**	**<0.001**	**<0.001**
Elevation	R	0.04	**0.27**	**0.24**	-	**0.84**	**-0.57**
	p-value	0.632	**0.001**	**0.004**	-	**<0.001**	**<0.001**
Habitat	R	0.10	**0.34**	**0.32**	**0.84**	-	**-0.92**
	p-value	0.239	**<0.001**	**<0.001**	**<0.001**	-	**<0.001**
Light Conditions	R	-0.12	**-0.34**	**-0.32**	**-0.57**	**-0.92**	-
	p-value	0.146	**<0.001**	**<0.001**	**<0.001**	**<0.001**	-

Table 10. Correlations of *L. superbum* for height, number of whorls, health, elevation, and light conditions. Bold indicates a correlation that was significant at the 0.01 level. Pearson's correlation coefficient (two-tailed) represented by R. Tests that could not be computed because the variable is constant are represented by a B. N = 225 for *L. superbum*. Bold indicates a significant correlation, where $p < 0.05$.

		Height	Number of Whorls	Health	Elevation	Light Conditions
Height	R	-	**0.93**	**0.24**	**0.27**	**-0.41**
	p-value	-	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Number of Whorls	R	**0.93**	-	**0.21**	**0.33**	**-0.40**
	p-value	**<0.001**	-	**0.001**	**<0.001**	**<0.001**
Health	R	**0.24**	**0.21**	-	**0.20**	**-0.18**
	p-value	**<0.001**	**0.001**	-	**0.002**	**0.007**
Elevation	R	**0.270**	**0.33**	**0.20**	-	**-0.41**
	p-value	**<0.001**	**<0.001**	**0.002**	-	**<0.001**
Light Conditions	R	**-0.41**	**-0.40**	**-0.18**	**-0.41**	-
	p-value	**<0.001**	**<0.001**	**0.007**	**<0.001**	-

Comparing Health among populations for *L. grayi* and *L. superbum* with Kruskal-Wallis Testing

The health score differed significantly between populations of *L. grayi* and *L. superbum* $p < 0.001$ for both species. For *L. grayi*, lilies on Roan Mountain (the Cloudland Hotel site) and Grassy Ridge were healthier than those in Blue Ridge Parkway (BRP) population 1 (BRP1), BRP2, and Whitetop Mountain (Table 11; Figure 5A). For *L. superbum*, lilies on Holston Mountain were healthier than those in Roan Mountain State Park and Unaka Mountain (Table 12; Figure 5B).

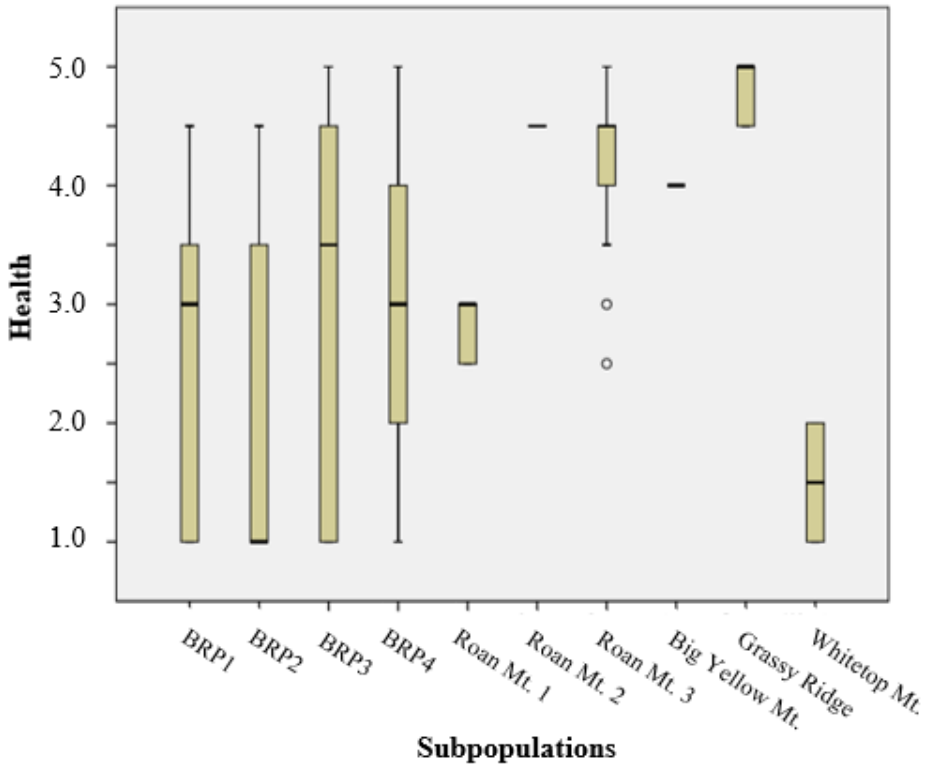
Table 11. Comparison of mean health among *L. grayi* populations. Means with the same letter were not significantly different. For significant differences, all p-values were less than 0.01.

Population	**Big Yellow Mt.**	**BRP1**	**BRP2**	**BRP3**	**BRP4**	**Roan Mt.**	**Grassy Ridge**	**Whitetop Mt.**
Mean health	$4.0^{A\,B}$	2.5^{B}	2.0^{B}	$2.8^{B\,C}$	$2.9^{A\,B}$	$4.0^{A\,C}$	$4.8^{A\,C}$	1.5^{B}

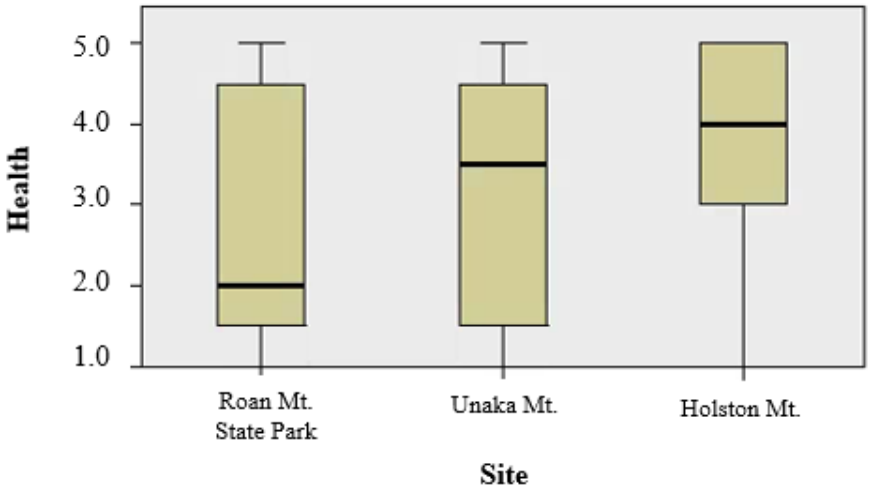
Table 12. Comparison of mean health among *L. superbum* populations. Means with the same letter were not significantly different. For significant differences, all p-values were less than 0.01.

Population	**Holston Mt.**	**Roan Mt. State Park**	**Unaka Mt.**
Mean health	3.8^{A}	2.7^{B}	3.0^{B}

A) *Lilium grayi*.



B) *Lilium superbum*.



C) Comparison of health among *L. superbum* subpopulations in 2015.

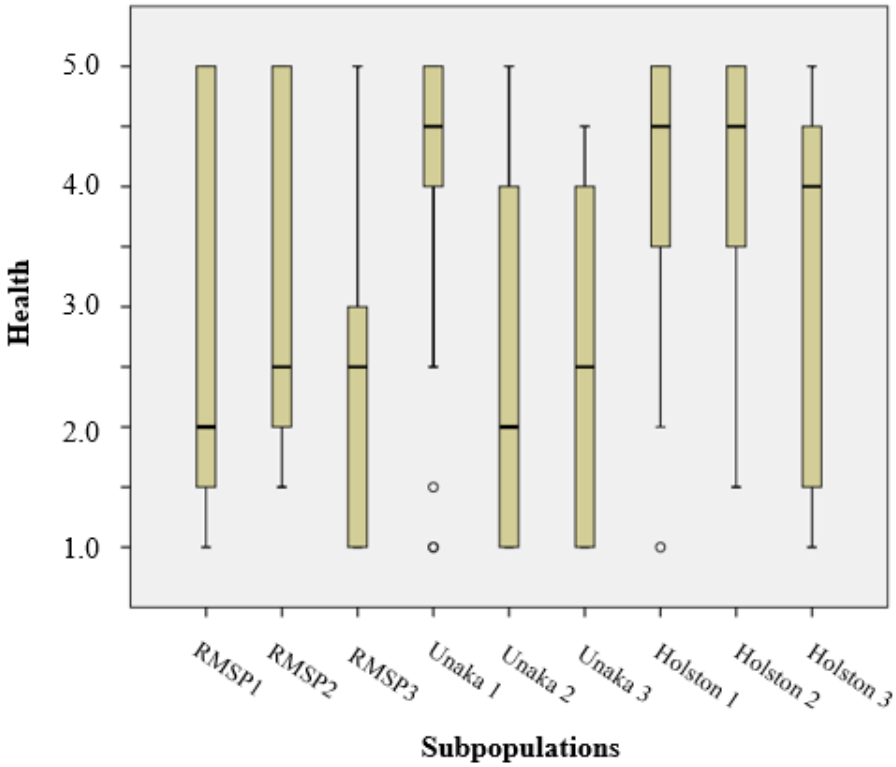


Figure 5. Health score of populations in 2015. Bars extending below and above the box represent the range of the health score. The heavy line represents the median. The extent of the box above and below the heavy line represents the 75th and 25th percentiles, respectively. Outliers (1.5–3x the interquartile range) are represented by a circle.

Comparing Health among Conditions: Plant Structure, Light Condition, and Elevation for both *L. grayi* and *L. superbum*

To test for a relationship between health and morphological variables in *L. grayi* and *L. superbum* the Kruskal-Wallis was used. Taller *L. grayi* (>45 cm) were significantly more healthy than smaller plants (≤45 cm); similiarly, larger *L. superbum* individuals (>76 cm) were also healthier than smaller ones (≤76 cm). Individuals of both species growing in full sun were significantly more healthy than those in shade. *Lilium grayi* growing at high elevations (>1372

meters or >4,500 feet) were significantly more healthy than those found in mid elevations (427–1372 meters or 1,400–4,500 feet); however, there was no significant difference between *L. superbum* individuals growing at mid elevations (427–1372 meters or 1,400–4,500 feet). A summary of all test statistics, p-values, and sample sizes for *L. grayi* are found in Table 13, and Table 14 has corresponding results for *L. superbum*.

Table 13. Health of *L. grayi* among populations, habitats, light conditions, elevation, and plant stature. Variables underlined indicate the healthier of the two conditions. Superscripts indicate statistical test utilized: [A]Kruskal-Wallis Test; [B]Pair-wise comparison. Figure refers to the corresponding boxplot associated with the statistical test. N = 148 for *L. grayi*.

Health Comparison	Test Statistic	Degrees of Freedom	P	Figure
Among Populations	37.2[A]	7	<0.001	3 A
Among Habitats	16.2[A]	2	<0.001	4
Forested Seep - Bald Habitat	-42.5[B]	1	<0.001	4
Forest - Bald Habitat	-36.0[B]	1	<0.001	4
Among Light Conditions: Full Sun – Shade	15.6[A]	1	<0.001	5 A
Elevation: High – Mid	7.7[A]	1	0.005	6
Stature: Larger – Smaller	34.8[A]	1	<0.001	7 A

Table 14. Comparison of health of *L. superbum* among populations, habitats, light conditions, elevation, and plant stature. Variables underlined indicate the healthier of the two conditions. Superscripts indicate statistical test utilized: [A]Kruskal-Wallis Test. Figure refers to the corresponding boxplot associated with the statistical test. N=225 for *L. superbum*.

Health Comparison	Test Statistic	Degrees of Freedom	P	Figure
Among Populations	17.9 [A]	2	<0.001	3 B
Among Light Conditions: Full Sun – Shade	7.0 [A]	1	0.008	5 B
Stature: Larger – Smaller	14.5[A]	1	<0.001	7 B

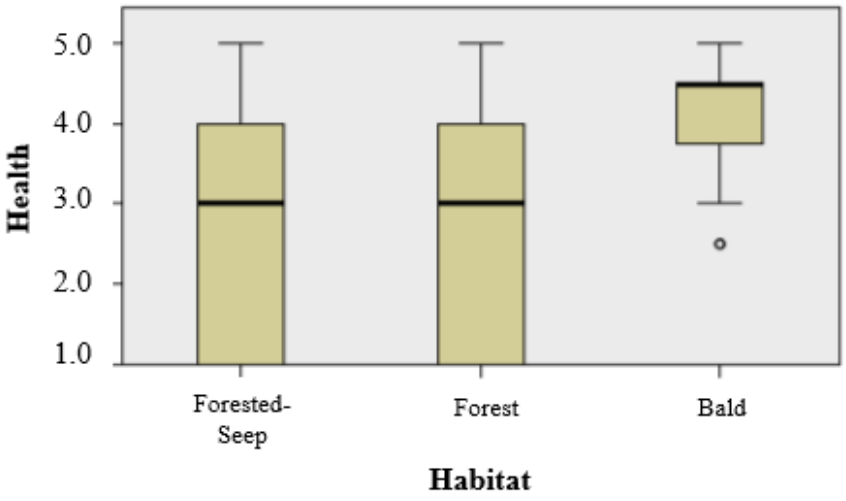


Figure 6. Comparison of health by habitat for *L. grayi* in 2015. Bars extending below and above the box represent the range of the health score. The heavy line represents the median. The extent of the box above and below the heavy line represents the 75th and 25th percentiles, respectively. Outliers (1.5–3x the interquartile range) are represented by a circle.

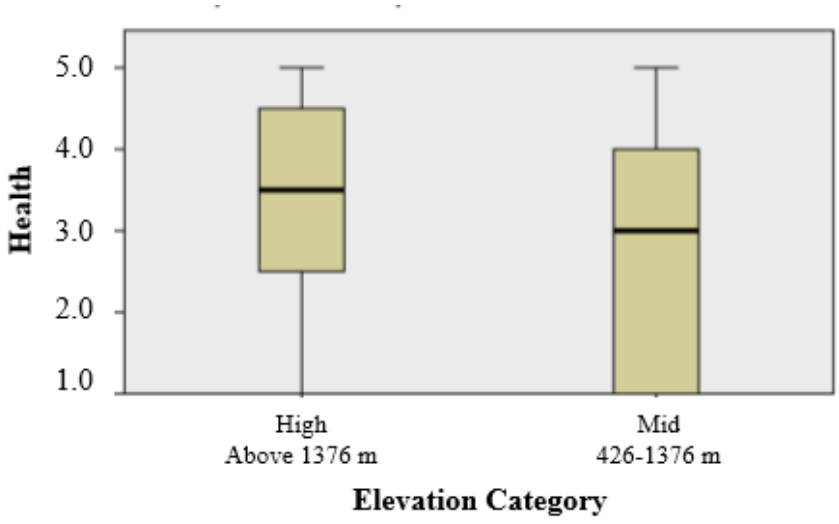
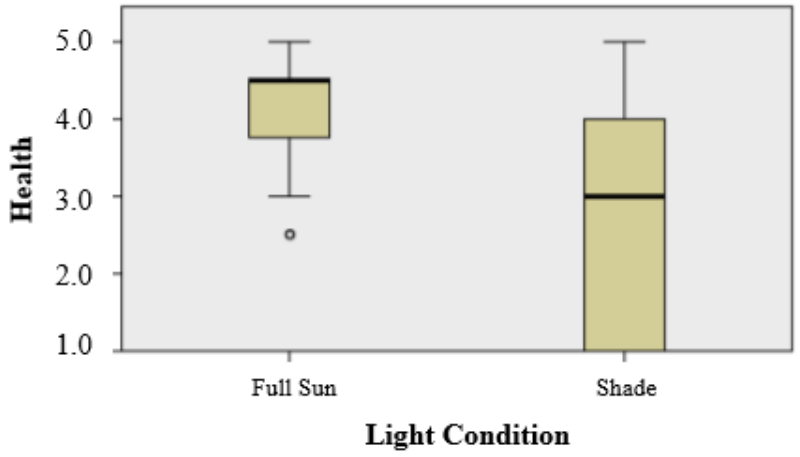


Figure 7. Health by elevation for *L. grayi* in 2015. Bars extending below and above the box represent the range of the health score. The heavy line represents the median. The extent of the box above and below the heavy line represents the 75th and 25th percentiles, respectively.

A) *Lilium grayi*.



B) *Lilium superbum*.

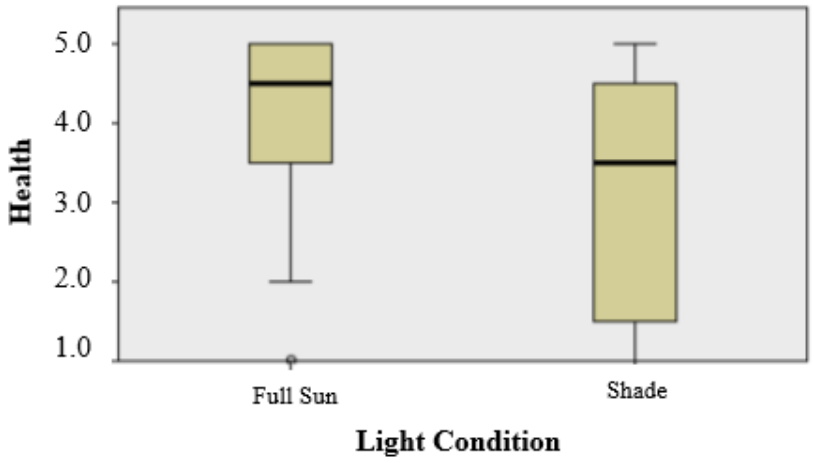
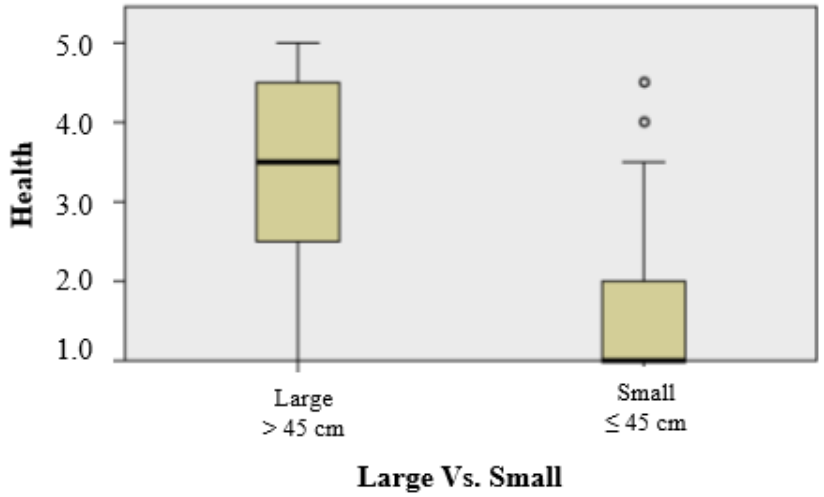


Figure 8. Health by light condition in 2015. Bars extending below and above the box represent the range of the health score. The heavy line represents the median. The extent of the box above and below the heavy line represents the 75th and 25th percentiles, respectively. Outliers (1.5–3x the interquartile range) are represented by a circle.

A) *Lilium grayi*.



B) *Lilium superbum*.

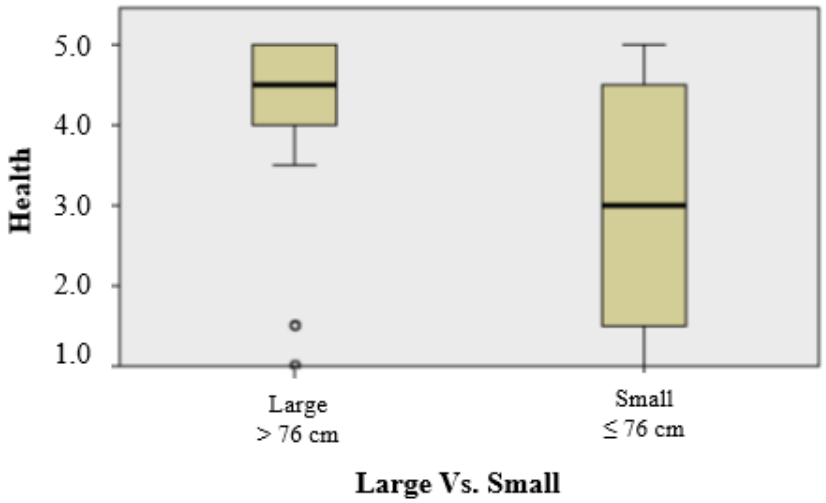


Figure 9. Health by plant stature (small vs. large). Bars extending below and above the box represent the range of the health score. The heavy line represents the median. The extent of the box above and below the heavy line represents the 75th and 25th percentiles, respectively. Outliers (1.5–3x the interquartile range) are represented by a circle.

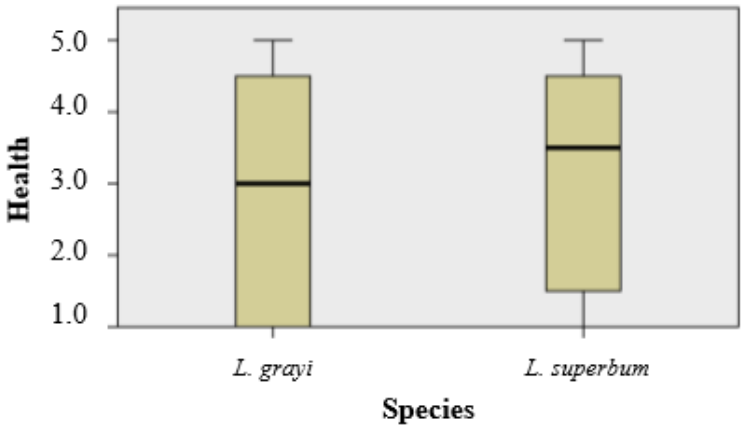
Comparing Demographic Characteristics between *L. grayi* and *L. superbum*

Several demographic characteristics differed significantly between the *L. grayi* and *L. superbum*. The average *L. grayi* tended to be taller than *L. superbum* (Table 15, Figure 10 B). The health score also differed significantly between species, with *L. superbum* having a higher health score than *L. grayi* (Table 15; Figure 10 A). Frequency histograms showing plant height, number of whorls, number of flowers, and health categories for both *L. grayi* and *L. superbum* in 2015 are in Figure 11.

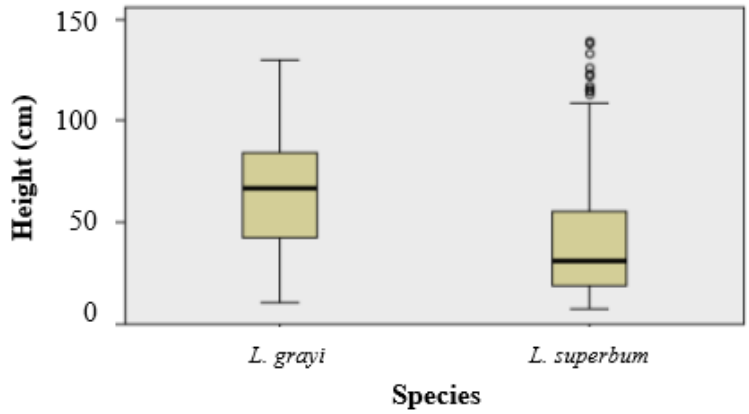
Table 15. Comparisons between *L. grayi* and *L. superbum* for health, height, and number of whorls. Variables underlined indicate the higher health mean and healthier of the two conditions. Superscripts indicate statistical test utilized: [A]Kruskal-Wallis Test. Figure refers to the corresponding boxplot associated with the statistical test. N = 148 in *L. grayi*; 225 in *L. superbum*.

Variables	Test Statistic	Degrees of Freedom	P	Figure
Health: *L. superbum* - *L. grayi*	7.6 [A]	1	0.006	10 A
Height: *L. superbum* - *L. grayi*	61.8 [A]	1	<0.001	10 B
Number of Whorls: *L. superbum* - *L. grayi*	41.1 [A]	1	<0.001	10 C

A) Health.



B) Height.



C) Number of whorls.

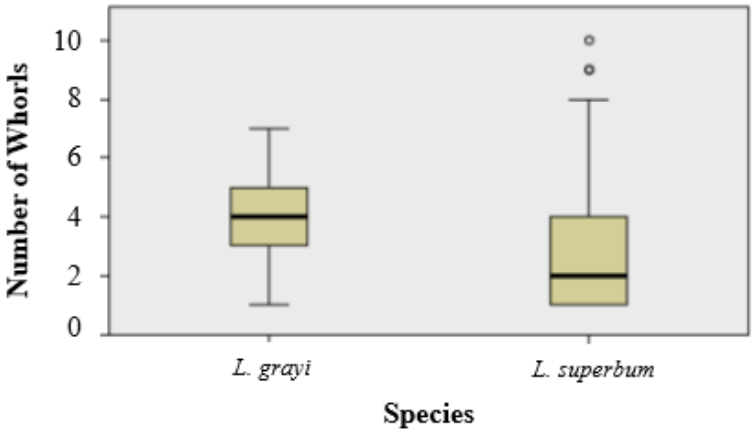
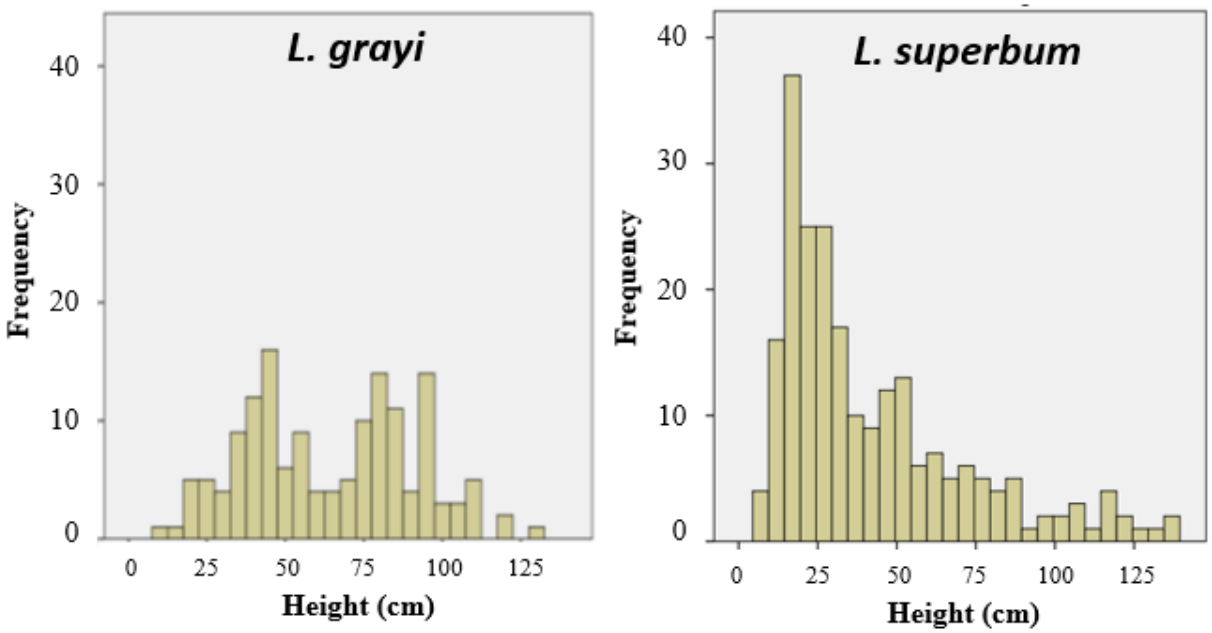
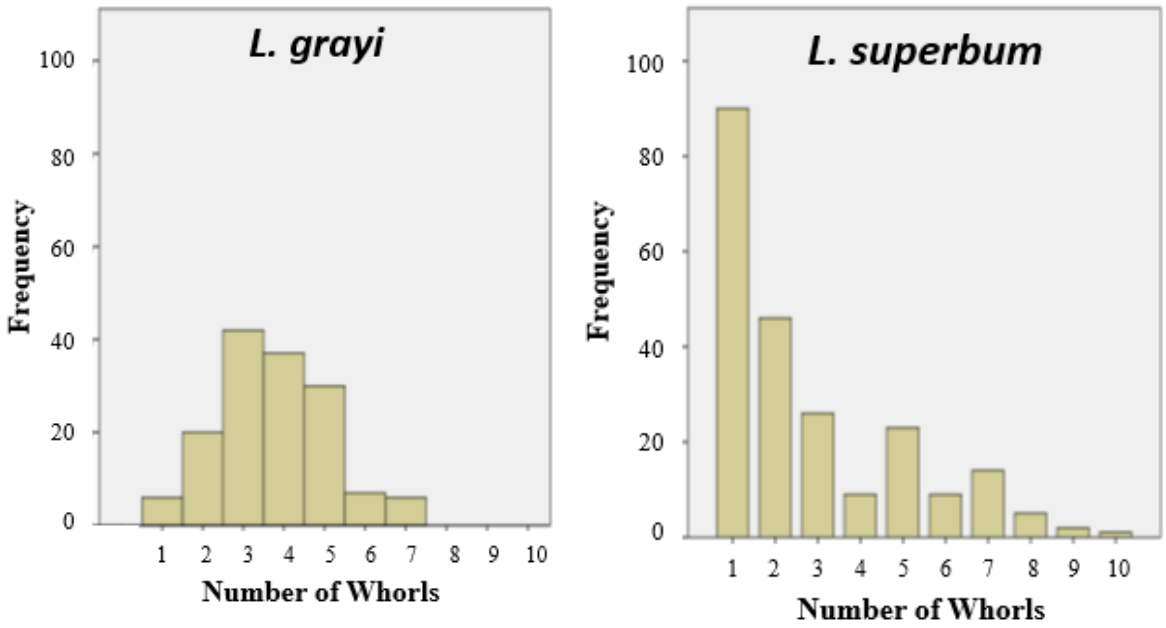


Figure 10. Species comparison of height, health, and whorls. Bars extending below and above the box represent the range of the health score. The heavy line represents the median. The extent of the box above and below the heavy line represents the 75th and 25th percentiles, respectively. Outliers (1.5–3x the interquartile range) are represented by a circle.

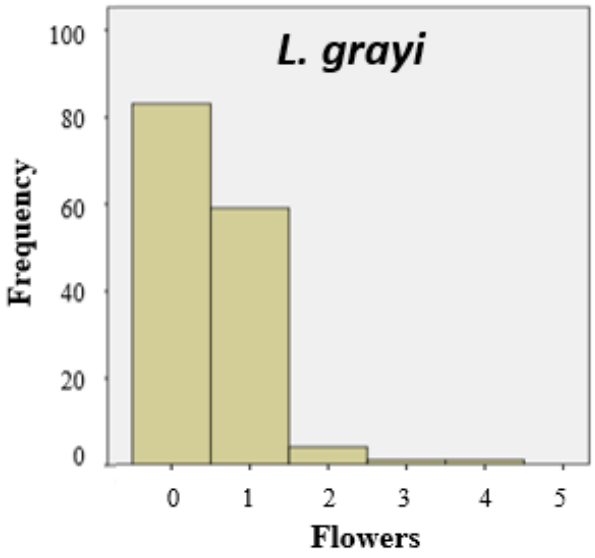
A) Height (cm).



B) Number of whorls.



C) Number of *L. grayi* flowers. Due to time and monetary constraints, priority was given to *L. grayi*, so monitoring of *L. superbum* flowering was not possible.



D) Health.

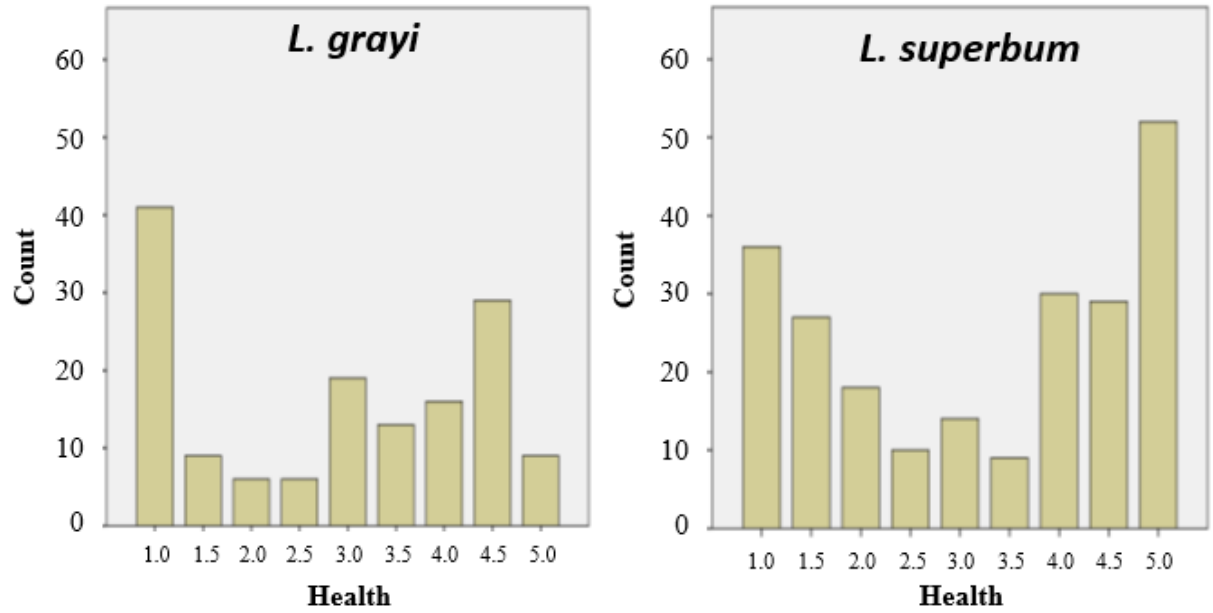
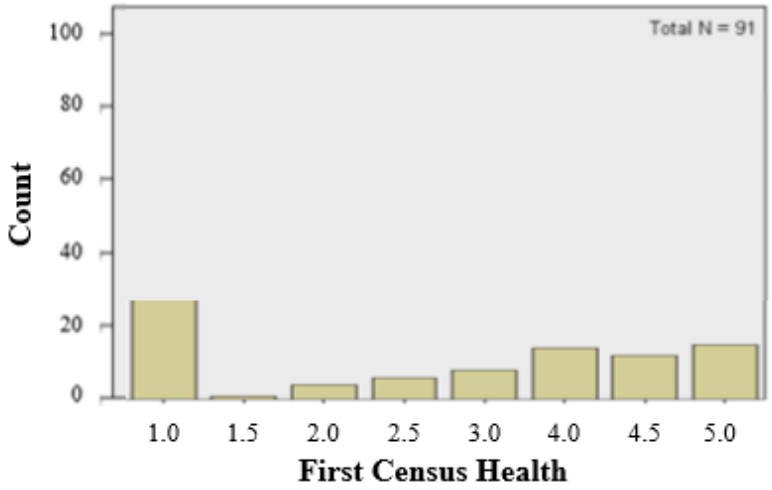


Figure 11. Frequency histograms showing plant height, number of whorls, number of flowers, and health categories for *L. grayi* and *L. superbum* in 2015.

In-season Health of *L. grayi*

The health scores decreased significantly between the first and second census using the Friedman's test (χ^2 (1) = 57.00, p <0.001) (Figure 12 A, B).

A) First census.



B) Second census.

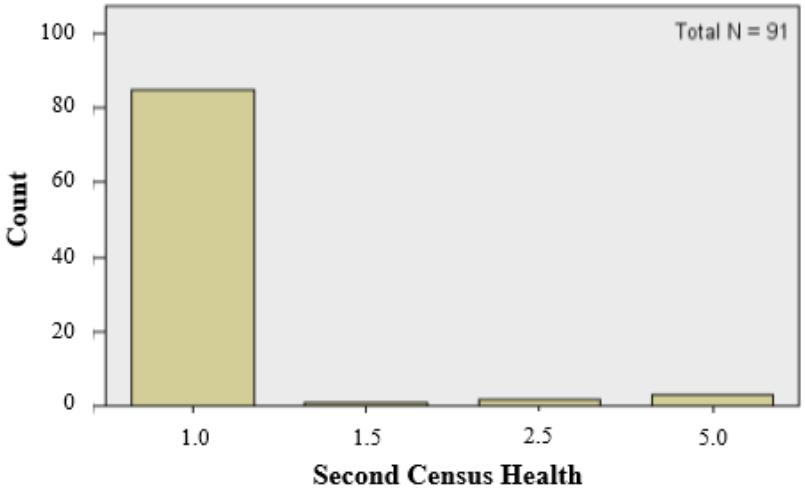


Figure 12. Distribution of *L. grayi* health at first and second census.

Between Season Health of *L. grayi*

The health of 232 *L. grayi* individuals did not differ significantly between years H (1) = 0.14, p = 0.71 (Figure 13).

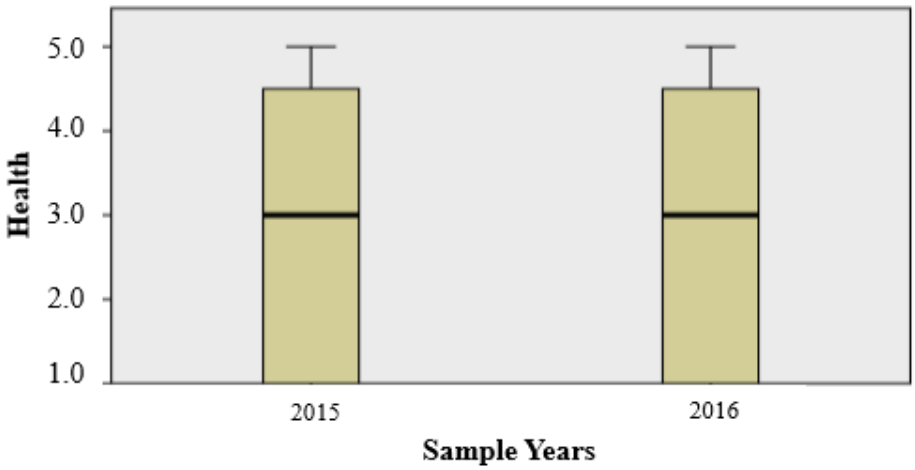


Figure 13. Comparison of *L. grayi* health between sample years.

Demography and Flowers

Comparing Flowers among Conditions: Population Site, Plant Structure, Light Condition, and Elevation for both *L. grayi* and *L. superbum*

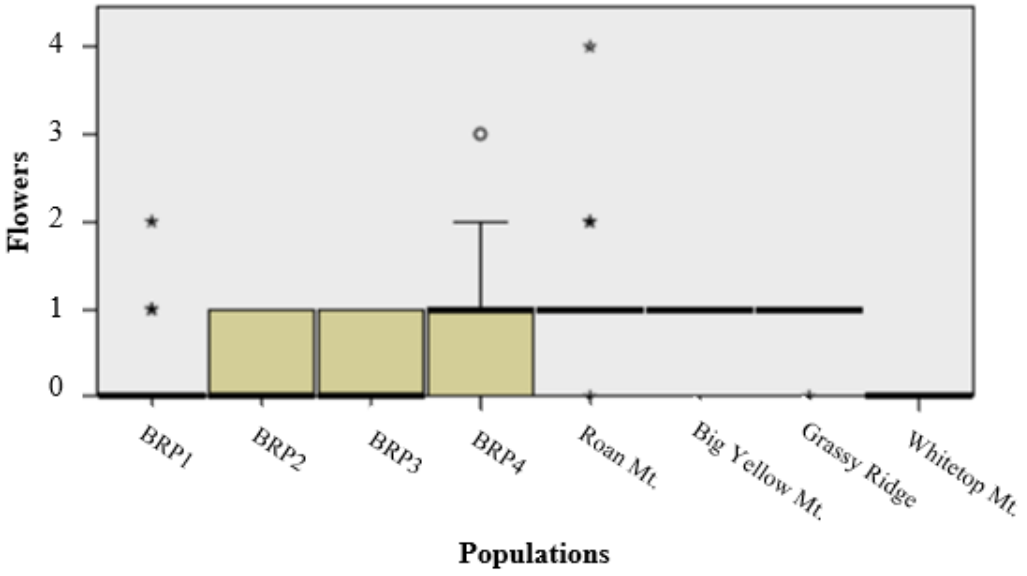
In *L. grayi*, the number of flowers per plant differed significantly between populations (Table 16; Figure 14 A) with more flowers on Roan Mountain compared to Whitetop Mountain and BRP populations 1, 2, and 3. There were also more flowers in BRP4 than BRP1 (Figure 14 A). Habitat type also influenced the number of flowers per plant (Figure 14 C). Lilies growing

in bald habitats had more flowers than those growing in forests and forested-seeps and lilies growing in full sun flowered more than those growing in shade (Table 16; Figure 14 C).

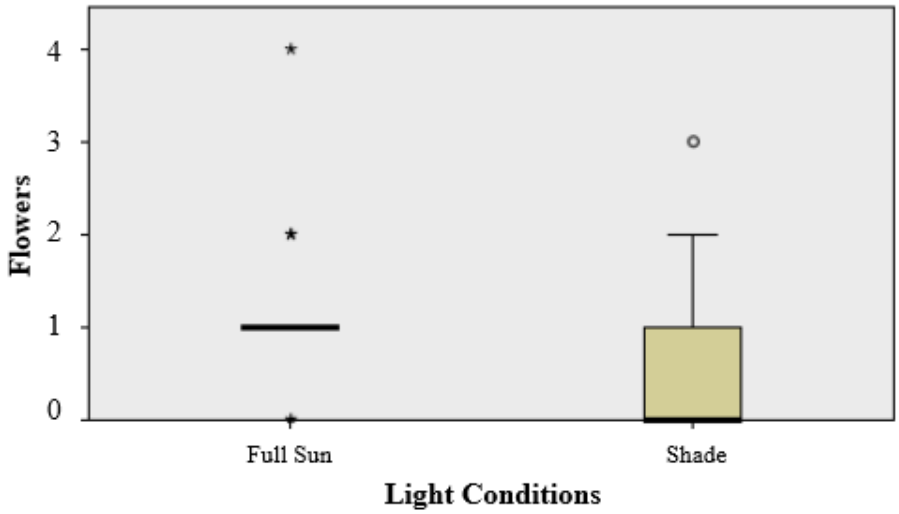
Table 16. Summary of nonparametric tests comparing flower number in *L. grayi*. Comparisons were among populations, light conditions, and habitats. Variables underlined indicate which condition had more flowers. Superscripts indicate statistical test utilized: [A]Kruskal-Wallis Test; [B]Pair-wise comparison. Figure refers to the corresponding boxplot associated with the statistical test.

Flower Number Comparison	Sample Size	Test Statistic	Degrees of Freedom	P	Figure
Among Populations	148	43.72 [A]	7	<0.001	11 A
Among Light Conditions: Full Sun – Shade	148	24.86 [A]	1	<0.001	11 B
Among Habitats	148	27.13 [A]	2	<0.001	11 C
Forests -Bald		-54.80 [B]	1	<0.001	11 C
Forested-Seeps -Bald		-43.07 [B]	1	<0.001	11 C

A) Comparison of *L. grayi* flower number by population in 2015.



B) Comparison of *L. grayi* flower number by light condition in 2015.



C) Comparison of *L. grayi* flower number by habitat in 2015.

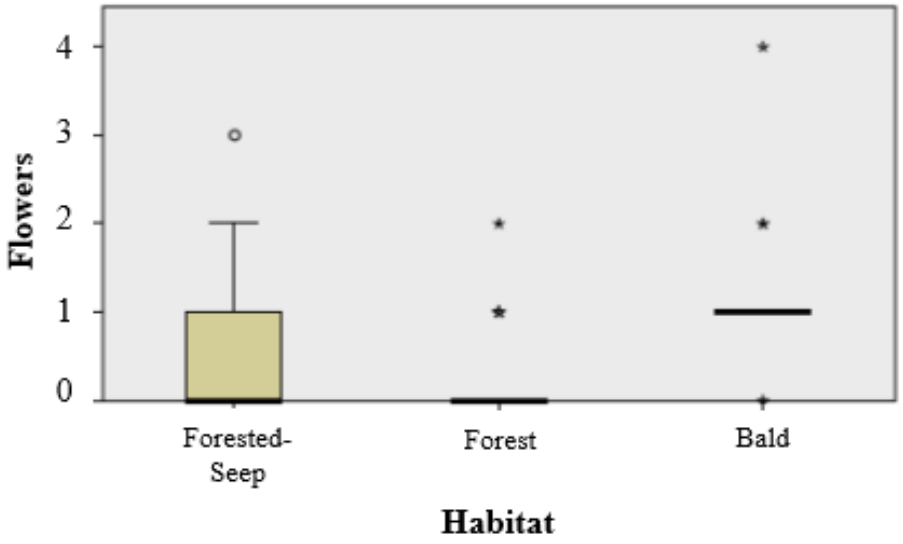
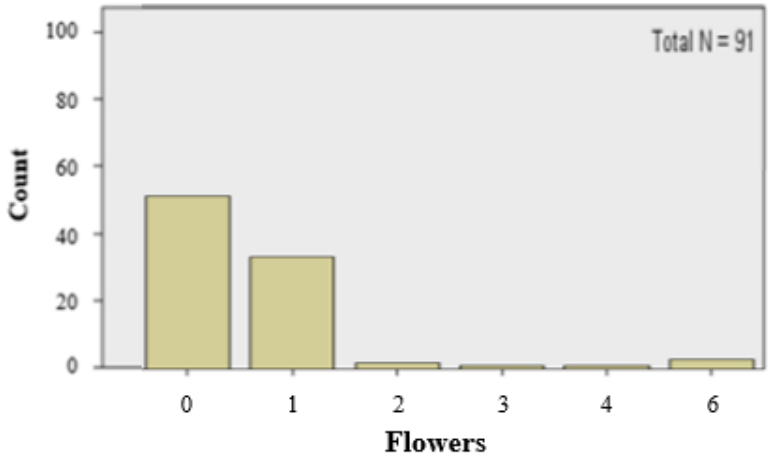


Figure 14. Comparison of *L. grayi* flower number by population, light condition, and habitat. Bars extending below and above the box represent the range of the health score. The heavy line represents the median. The extent of the box above and below the heavy line represents the 75th and 25th percentiles, respectively. Outliers (1.5–3x the interquartile range) are represented by a circle, and extreme values (>3x the interquartile range) are represented by a star.

Lilium grayi reproduction

The number of capsules produced was significantly less than the number of flowers recorded in the first census χ^2 (1) = 37.00, p <0.001 (Figure 15 A, B). Only sites with first and second census data were included in this analysis (BRP1, Elk Hollow Preserve, Roan Mountain (Cloudland Hotel Site), and Whitetop populations).

A) Flower distribution from first census.



B) Capsule distribution from second census.

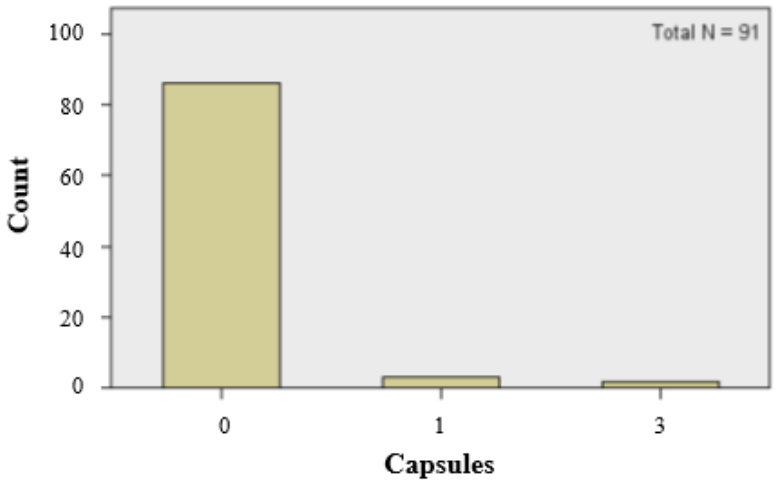


Figure 15. *Lilium grayi* flower histogram from first census and capsule histogram from second census.

Fungal Inoculation Trials – Lesion Analysis

Fungal Lesion Development

Whether an abrasion develops a lesion does not depend on the species inoculated using a Fisher's exact test ($p = 0.55$), and abrasion development does not depend on inoculum type ($x^2 (1) = 0.53$, $p = 0.47$).

Fungal Lesion Size Analysis. Abrasions without Lesion Development Excluded.

Assumption Testing. To determine if a difference in lesion size area was seen over the course of approximately 30 days post infection with *P. inconspicua*, a two-way repeated measures ANOVA was conducted. There were 12 outliers identified (Figure 16). The data was not normally distributed for all four time points, as determined by the Shapiro-Wilk test ($p < 0.05$). This represents a violation of two of the main assumptions of the repeated-measures ANOVA (Lund 2015). The assumption of sphericity was also violated, $\chi^2 (2) = 18.69$, $p = 0.002$. A result that means the variance is not equal between the levels of the within-subject factor [the time point 1-4]; violating this assumption is very common in repeated-measure ANOVAs (Lund 2015). To remedy this violation, a Greenhouse-Geisser correction was used ($\varepsilon = 0.70$), which adjusts the degrees of freedom and allows for a correct p-value to be reported (Lund 2015).

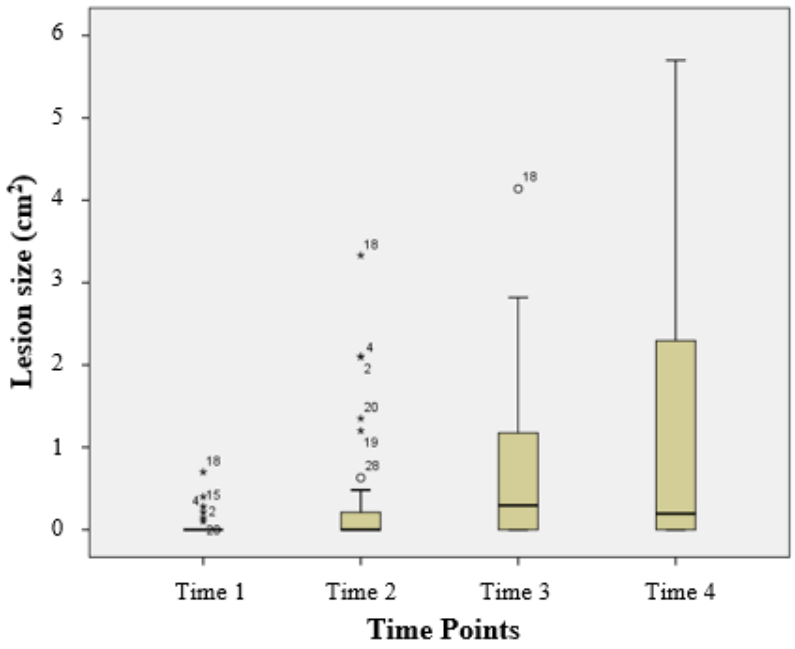


Figure 16. Comparison of lesion size by time period for all three fungal lesion experiments. Bars extending below and above the box represent the range of the lesion area size in cm^2. The heavy line represents the median. The extent of the box above and below the heavy line represents the 75^{th} and 25^{th} percentiles, respectively. Outliers (1.5–3x the interquartile range) are represented by a circle, and extreme values (>3x the interquartile range) are represented by a star.

Test Results. Lesion size increased significantly though time (Table 17; Figure 17). The rate increase was dependent on the inoculum type and the species inoculated, as a significant inoculum type by species interaction for mean lesion size over time was shown (Table 17). Lesion change over time was a significant, but an expected result, as fungal lesions expand as infection progresses. The significant interactions of inoculum and species are found in Table 20. Figures 18 and 19 show lesion size over time by inoculum type and species inoculated, respectively. Significant p values for lesion size over time by inoculum type are located in Table 18 and the corresponding table for lesion size over time by species is found in Table 19.

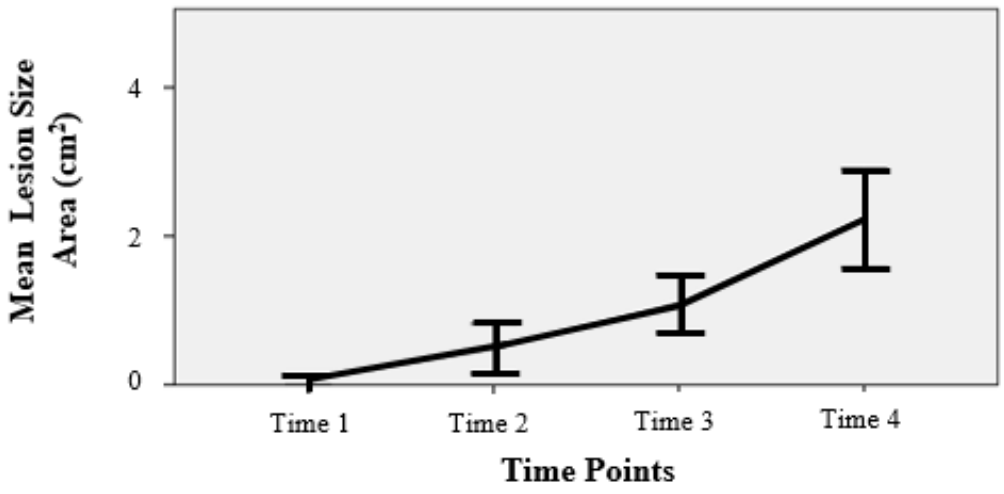


Figure 17. Mean lesion size (cm) across time points 1–4 for all fungal inoculation experiments. Bars represent error bars with 95% Confidence Interval.

Table 17. Summary repeated multivariate ANOVAs for lesion size over time for different inoculum types used on three species. Significant p-values, based on Wilks lambda, are in bold.

Lesion Size Effects	Numerator DF	Denominator DF	Test statistic	P
Time	3	18	47.61	**<0.001**
Time x inoculum type	3	18	8.59	**<0.001**
Time x species	6	36	2.35	**0.05**
Time x inoculum type x species	6	36	2.94	**0.02**

Table 18. Least squares mean lesion size (LS mean) by type of *P. inconspicua* inoculum. Superscripts of the same letter indicate an insignificant p-value among mean lesion sizes within that time period. Tukey post hoc tests were used to determine p-values. No significant results were found, $p > 0.05$.

Inoculum		**Hyphal culture from *L. grayi***	**Conidia from *L. superbum***
LS Mean	Time 1: Day 3-9	0.07[A]	0.00 [A]
	Time 2: Day 10-14	0.52 [A]	0.11 [A]
	Time 3: Day 15-20	1.3 [A]*	0.60 [A]*
	Time 4: Day 21-32	1.59 [A]	2.40 [A]

*The inoculum types in time period thee had insignificant but borderline p-value of 0.06.

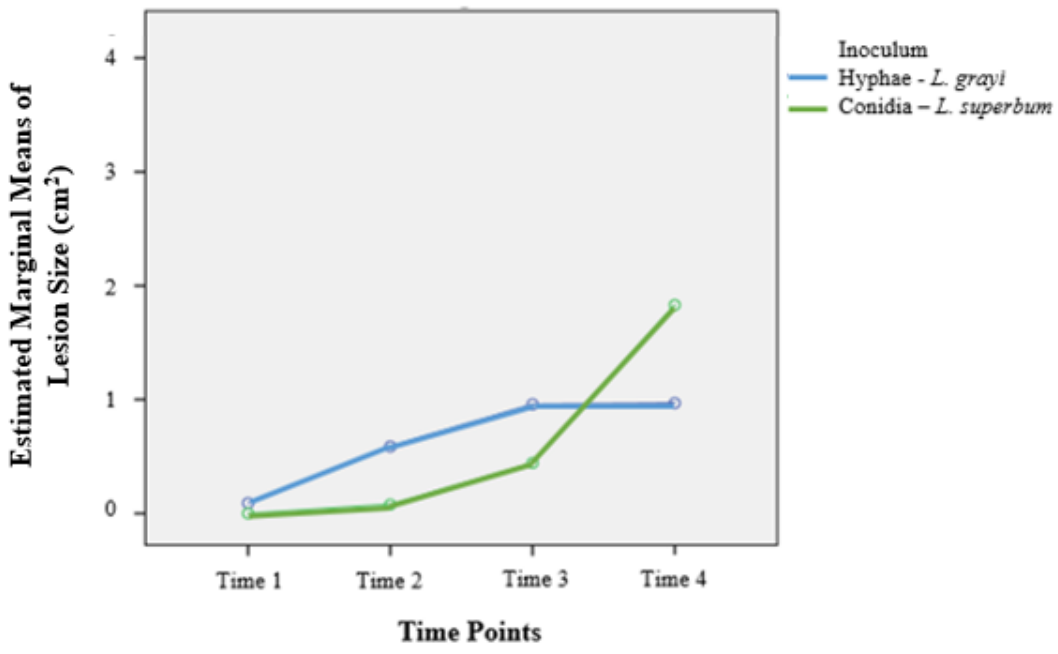


Figure 18. Lesion size by inoculum type—*P. inconspicua* hyphae from *L. grayi* and *P. inconspicua* conidia from *L. superbum*.

Table 19. Least squares mean lesion size (LS mean) by species. Superscripts of the same letter indicate an insignificant p-value among mean lesion sizes within that time period. Tukey post hoc tests were used to determine p-values. No significant results were found, $p > 0.05$.

Species		***L. grayi***	***L. michauxii***	***L. superbum***
LS Mean	Time 1: Day 3-9	0.00^{A}	0.00^{A}	0.10^{A}
	Time 2: Day 10-14	0.18^{A}	0.19^{A}	0.58^{A}
	Time 3: Day 15-20	0.63^{A}	1.24^{A}	0.97^{A}
	Time 4: Day 21-32	1.2^{A}*	3.00^{A}*	1.80^{A}

*The *L. grayi* and *L. michauxii* in time period four had an insignificant but borderline p-value of 0.054.

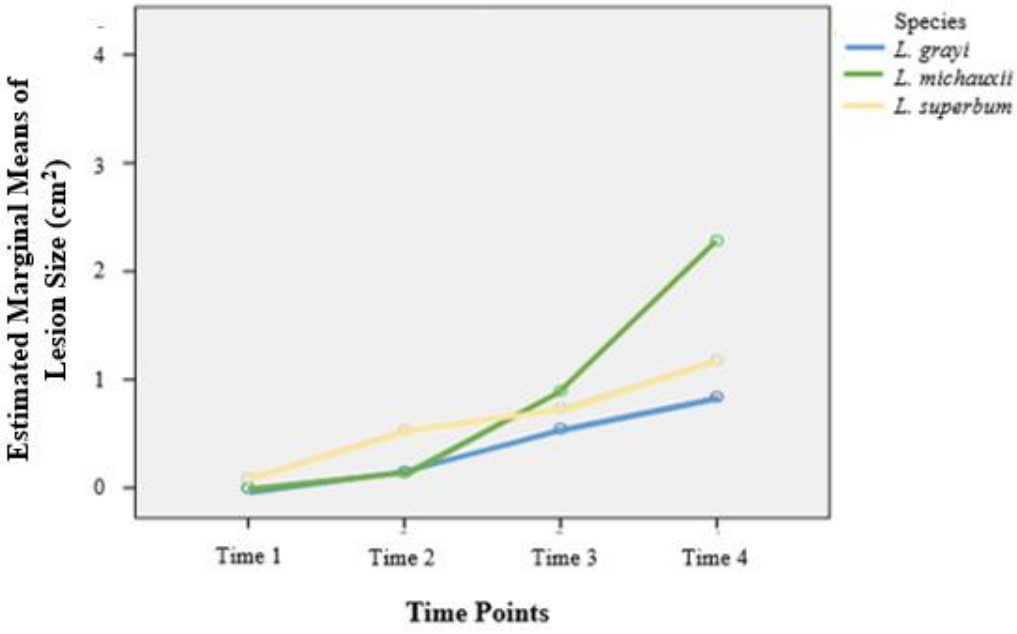


Figure 19. Lesion size by species inoculated—*L. grayi*, *L. michauxii*, and *L. superbum*.

Table 20. Comparison of least squares mean lesion size (LS Mean) for each inoculum and species combination. Inoculum types: 1 = *P. inconspicua* hyphae from *L. grayi*; 2 = *P. inconspicua* conidia from *L. superbum*. Means with the same superscript indicate an insignificant p-value within that time period. Tukey post hoc tests were used to determine p-values.

Inoculum*species		**1*L. grayi**	**1*L. michauxii**	**1*L. superbum**	**2*L. grayi**	**2*L. michauxii**	**2*L. superbum**
LS Mean	Time 1: Day 3-9	0.00 ^{A}	0.00 ^{A}	0.20 ^{A}	0.00 ^{A}	0.00 ^{A}	0.00 ^{A}
	Time 2: Day 10-14	0.26 ^{A}	0.17 ^{A}	1.15 ^{A}	0.11 ^{A}	0.21 ^{A}	0.02 ^{A}
	Time 3: Day 15-20	0.72 ^{A}	1.38 ^{A}	**1.82** $^{A, B}$	0.55 ^{A}	1.11 ^{A}	**0.12** $^{A, C}$
	Time 4: Day 21-32	0.72 ^{A}	1.4 ^{A}	2.66 $^{A, C}$	1.68 ^{A}	**4.57** $^{B, C}$	0.94 ^{A}

Additional Analysis. Lesion analysis that included all lesion measurements (even abrasions that did not develop lesions) was conducted and yielded similar results (Table 21).

Table 21: Summary repeated multivariate ANOVAs for lesion size over time (abrasions that did not develop lesions were included) for different inoculum types used on three species. Significant p-values, based on Wilks lambda, are in bold.

Lesion Size Effects	**Numerator DF**	**Denominator DF**	**Test statistic**	**P**
Time	3	28	24.10	**<0.001**
Time x inoculum type	3	28	13.11	**<0.001**
Time x species	6	56	3.76	**0.003**
Time x inoculum x species	6	56	5.36	**<0.001**

Fungal Inoculation Trials – Area of Chlorotic Zone Analysis

Area of Chlorotic Zone

Assumption Testing. To determine if a significant difference in chlorosis size was seen over the course of approximately 30 days post infection with *P. inconspicua*, a one-way repeated measures ANOVA was conducted. There were seven outliers identified (Figure 20). The data was not normally distributed for all four time points, as determined by the Shapiro-Wilk test ($p < 0.05$). This represents a violation of two of the main assumptions of the repeated-measures ANOVA (Lund 2015). The assumption of sphericity was also violated, $\chi^2 (2) = 14.45$, $p = 0.01$. A result that means the variance is not equal between the levels of the within-subject factor [the time point 1-4]; violating this assumption is very common in repeated-measure ANOVAs (Lund 2015). To remedy this violation, a Greenhouse-Geisser correction was used ($\varepsilon = 0.81$), which adjusts the degrees of freedom and allows for a correct p-value to be reported (Lund 2015).

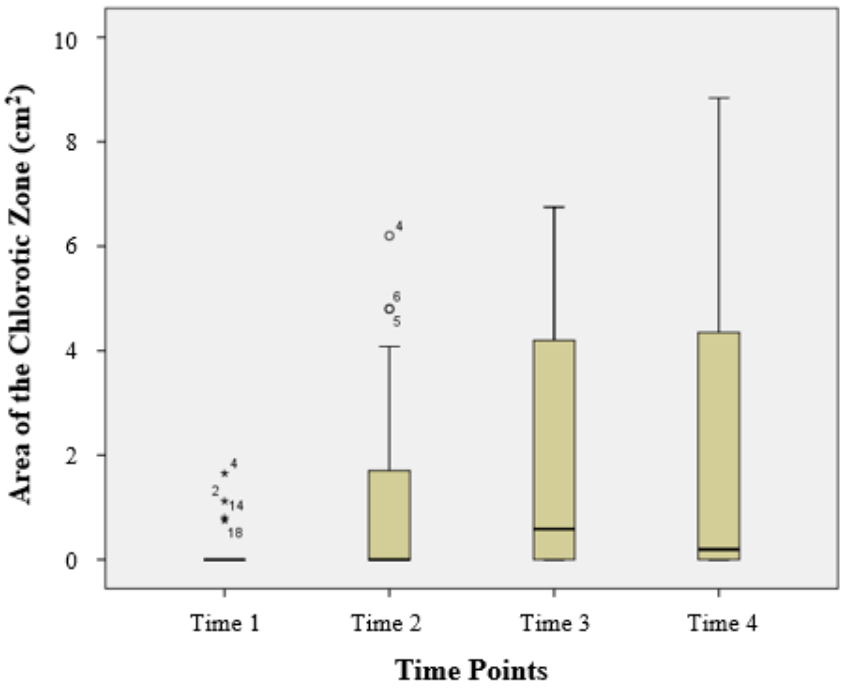


Figure 20. Comparison of chlorosis size by time period for all three fungal lesion experiments. Bars extending below and above the box represent the range of the chlorosis area size in cm^2. The heavy line represents the median. The extent of the box above and below the heavy line represents the 75^{th} and 25^{th} percentiles, respectively. Outliers (1.5–3x the interquartile range) are represented by a circle, and extreme values (>3x the interquartile range) are represented by a star.

Testing Results. The area of the chlorotic zone increased significantly over time (Table 22; Figure 21). There was a significant interaction of chlorosis size over time and species type inoculated: *L. grayi*, *L. michauxii*, or *L. superbum*. A difference in chlorosis least square mean was found for time period two and three between inoculum source, with *P. inconspicua* hyphal inoculum from a *L. grayi* individual producing larger chlorosis areas than the conidia *P. inconspicua* culture gathered from a *L. superbum*, depending on the time period (Table 23). *Lilium grayi* produced a larger chlorosis size than *L. michauxii* for time period two (Table 24).

For time period three, all three species differed from each other in chlorosis size, with *L. grayi* having the largest chlorosis and *L. superbum* having the least (Table 24). For the last time period, *L. superbum* had smaller lesions than the rest of the species (Table 24; Figure 23). The rate of increase in the chlorotic zone area depended on the inoculum type used and the species inoculated, as a significant inoculum type by species interaction for mean chlorosis size over time was found (Table 22; Table 25). These results parallel that of the lesions size analysis. Each result was calculated using Wilks' Lambda MANOVA.

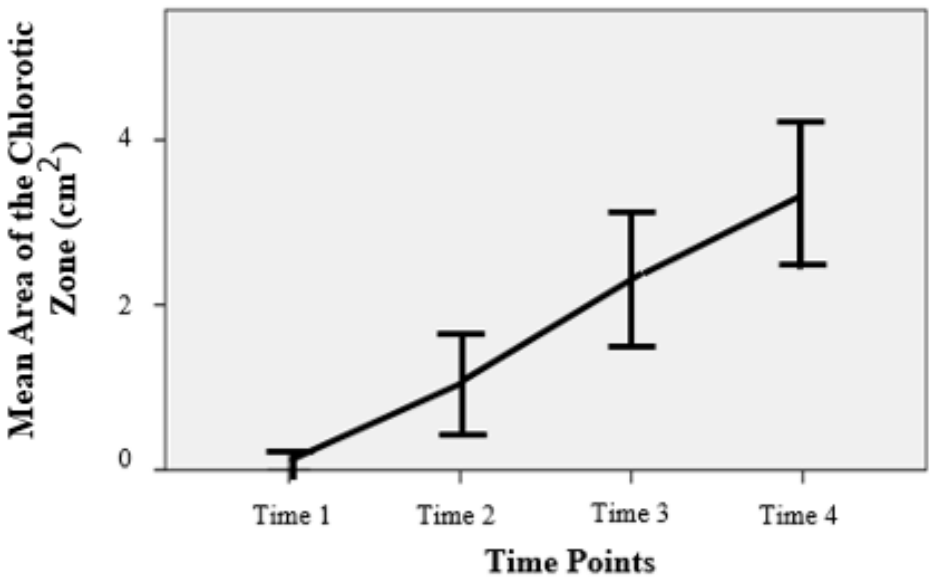


Figure 21. Mean area of the chlorosis zone (cm) across time points 1-4 for all fungal inoculation experiments. Bars represent error bars with 95% Confidence Interval.

Table 22. Summary repeated multivariate ANOVAs for area of the chlorotic zone over time for different inoculum types used on three species. Significant p-values, based on Wilks lambda, are in bold.

Source	Numerator DF	Denominator DF	Test statistic: F value	P
Time	3	19	172.72	**<0.001**
Time x inoculum	3	19	27.12	**<0.001**
Time x species	6	38	19.28	**<0.001**
Time x inoculum x species	6	38	18.46	**<0.001**

Table 23. Least squares mean chlorosis size (LS mean) by type of *P. inconspicua* inoculum. Superscripts of the same letter indicate an insignificant p-value among mean lesion sizes within that time period. Tukey post hoc tests were used to determine p-values. Significant results were found, $p < 0.001$.

Inoculum		Hyphal culture from *L. grayi*	Conidia from *L. superbum*
LS Mean	Time 1: Day 3-9	0.16 ^{A}	0.00 ^{A}
	Time 2: Day 10-14	**2.36** ^{A}	**0.00** ^{B}
	Time 3: Day 15-20	**4.81** ^{A}	**1.88** ^{B}
	Time 4: Day 21-32	4.99 ^{A}	4.56 ^{A}

Table 24. Least squares mean chlorosis size (LS mean) by species. Superscripts of the same letter indicate an insignificant p-value among mean chlorosis sizes within that time period. Tukey post hoc tests were used to determine p-values. Significant p-values were $p < 0.05$.

Species		*L. grayi*	*L. michauxii*	*L. superbum*
LS Mean	Time 1: Day 3-9	-0.00 ^{A}	-0.00 ^{A}	0.24 ^{A}
	Time 2: Day 10-14	**2.19** $^{A, C}$	**0.31** $^{B, C}$	1.04 ^{C}
	Time 3: Day 15-20	**4.88** ^{A}	**3.34** ^{B}	**1.78** ^{C}
	Time 4: Day 21-32	4.88 ^{A}	6.51 ^{A}	**2.99** ^{B}

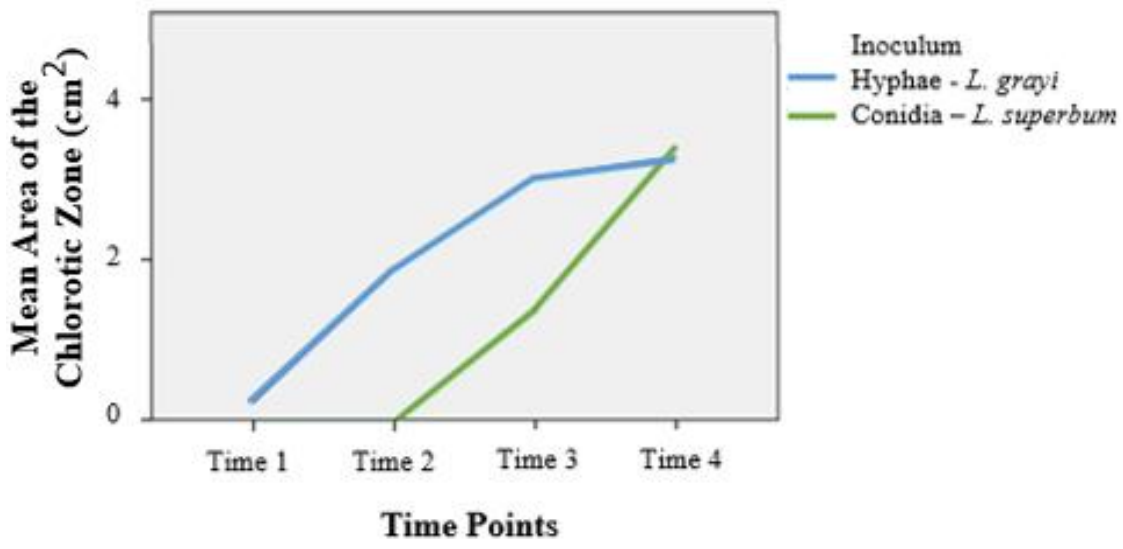


Figure 22. Area of chlorotic zone by inoculum type—*P. inconspicua* hyphae from *L. grayi* and *P. inconspicua* conidia from *L. superbum*.

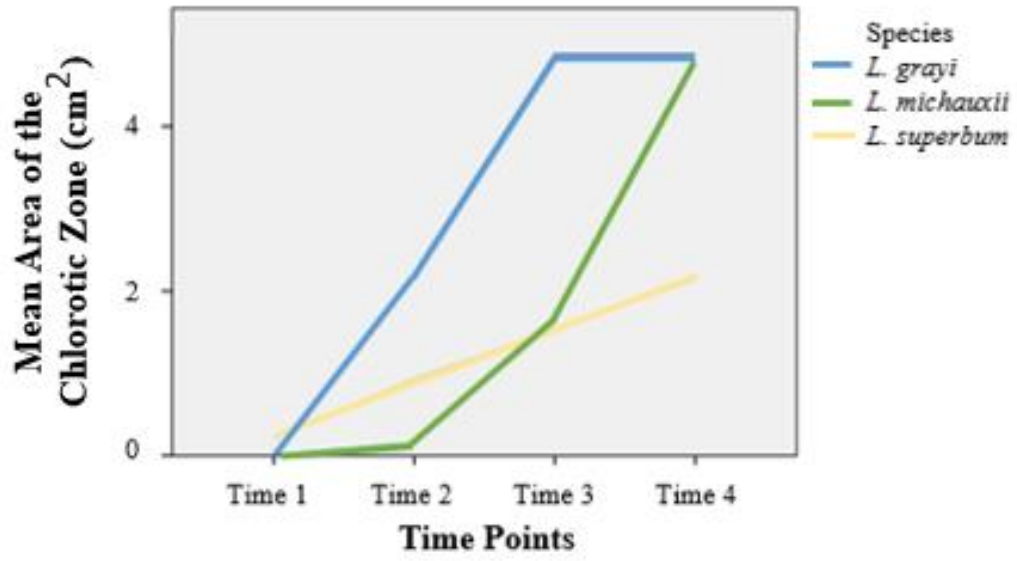


Figure 23. Chlorosis size by species inoculated—*L. grayi*, *L. michauxii*, and *L. superbum*.

Post hoc testing. To determine which inoculum type, species, or interaction differed, Tukey post hoc testing using pairwise comparisons was performed. A difference in chlorosis least square mean was found for time period two and three between inoculum sources, with *P. inconspicua* hyphal inoculum from a *L. grayi* individual producing larger chlorosis areas than the conidia *P. inconspicua* culture gathered from a *L. superbum* (Table 23; Figure 22). No difference among area of chlorotic zone was seen among the species in time period one (Table 24). *Lilium grayi* produced a larger chlorosis size than *L. michauxii* for time period two (Table 24). For time period three, all three species differed from each other in chlorosis size, with *L. grayi* having the largest chlorosis and *L. superbum* having the least (Table 24). For the last time period, *L. superbum* had smaller lesions than the rest of the species (Table 24; Figure 23). For the interaction of inoculum and species on chlorosis size, there were several significant findings (Table 25).

Table 25. Least squares mean chlorosis size (LS mean) by interaction of inoculum and species, where 1 = *P. inconspicua* inoculum hyphae from *L. grayi* and 2 = *P. inconspicua* conidia from *L. superbum*. Superscripts of the same letter indicate an insignificant p-value among mean chlorosis sizes within that time period. Tukey post hoc tests were used to determine p-values.

Inoculum*species		**1*L. grayi***	**1*L. michauxii***	**1*L. superbum***	**2*L. grayi***	**2*L. michauxii***	**2*L. superbum***
LS Mean	Time 1: Day 3-9	-0.00 ^{A}	-0.00 ^{A}	0.48 ^{A}	-0.00 ^{A}	-0.00 ^{A}	-0.00 ^{A}
	Time 2: Day 10-14	**4.38 ^{B}**	0.63 ^{A*}	2.07 ^{A}	0.00 ^{A}	0.00 ^{A}	0.00 ^{A}
	Time 3: Day 15-20	4.38 $^{A, B}$	6.75 ^{A}	3.28 $^{A, B}$	5.37 $^{A, B}$	**-0.00 ^{C}**	**0.27 ^{C}**
	Time 4: Day 21-32	4.38 $^{A, B}$	6.75 ^{A}	3.85 $^{A, B}$	5.37 ^{A}	6.30 ^{A}	**2.05 ^{B}**

*An insignificant but borderline p-value was found p = 0.08.

Additional Analysis. Area of chlorotic zone analysis that included all chlorosis measurements (even those that did not develop chlorosis) was conducted and yielded similar results (Table 26).

Table 26: Summary repeated multivariate ANOVAs for area of chlorotic zone over time (abrasions that did not develop lesions were included) for different inoculum types used on three species. Significant p-values, based on Wilks lambda, are in bold.

Lesion Size Effects	**Numerator DF**	**Denominator DF**	**Test statistic**	**P**
Time	3	28	33.50	**<0.001**
Time x inoculum type	3	28	21.87	**<0.001**
Time x species	6	56	11.97	**<0.001**
Time x inoculum x species	6	56	13.66	**<0.001**

Fungal Inoculation Trials – Incubation Period

Incubation period. Abrasion without lesion development excluded.

Assumption testing. To determine if a difference in incubation period was seen post infection with *P. inconspicua*, a two-way repeated measures ANOVA was conducted. There were four outliers identified (Figure 24). The data was normally distributed for all four time points, as determined by the Shapiro-Wilk test ($p = 0.09$). The assumption of homogeneity of variance was not violated, as determined by the Levene's test ($p = 0.16$) (Lund 2015).

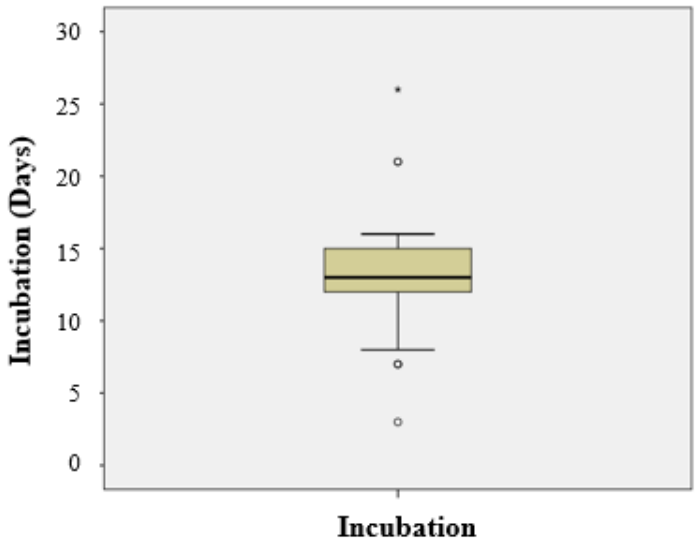


Figure 24. Comparison of incubation time (in days) for all three fungal lesion experiments. Bars extending below and above the box represent the range of incubation time. The heavy line represents the median. The extent of the box above and below the heavy line represents the 75th and 25th percentiles, respectively. Outliers (1.5–3x the interquartile range) are represented by a circle, and extreme values (>3x the interquartile range) are represented by a star.

Testing Results. Table 27 shows a summary of the descriptive statistics for incubation period for all three fungal inoculation trials. The incubation period for lesions to develop did not differ significantly among inoculum types or among species (Table 28; Figure 25; Figure 26).

Table 27. Descriptive statistics showing mean incubation days by inoculum type, species, and by interaction of inoculum and species. Inoculum types: 1 = *P. inconspicua* hyphae from *L. grayi*; 2 = *P. inconspicua* conidia from *L. superbum*. SD = standard deviation and N = sample size.

	Factors	**Mean incubation (days)**	**N**
Inoculum	Hyphal culture from *L. grayi*	11.96	14
	Conidia from *L. superbum*	15.33	12

Table 27. Continued.

	Factors	Mean incubation (days)	N
Species	*L. grayi*	13.08	7
	L. michauxii	13.75	6
	L. superbum	14.11	13
Interactions	1**L. grayi*	12.67	3
	1**L. michauxii*	13.00	2
	1**L. superbum*	10.22	9
	2**L. grayi*	13.5	4
	2**L. michauxii*	14.5	4
	2**L. superbum*	18.00	4

Table 28. Summary of two-way ANOVAs for incubation days by inoculum type, species, and by interaction of inoculum and species. No significant p-values were found.

	DF effect	DF error	Test statistic, F	P
Inoculum	1	20	2.91	0.10
Species	2	20	011	0.90
Inoculum*species	2	20	1.56	0.24

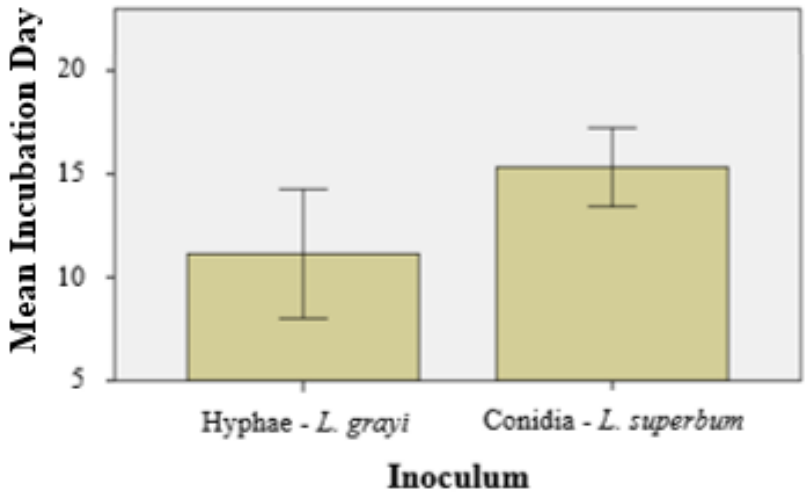


Figure 25. Comparison of incubation time (in days) for all three fungal lesion experiments by *P. inconspicua* inoculum type–*P. inconspicua* hyphae from *L. grayi* and *P. inconspicua* conidia from *L. superbum*. Bars represent 95% confidence interval (CI).

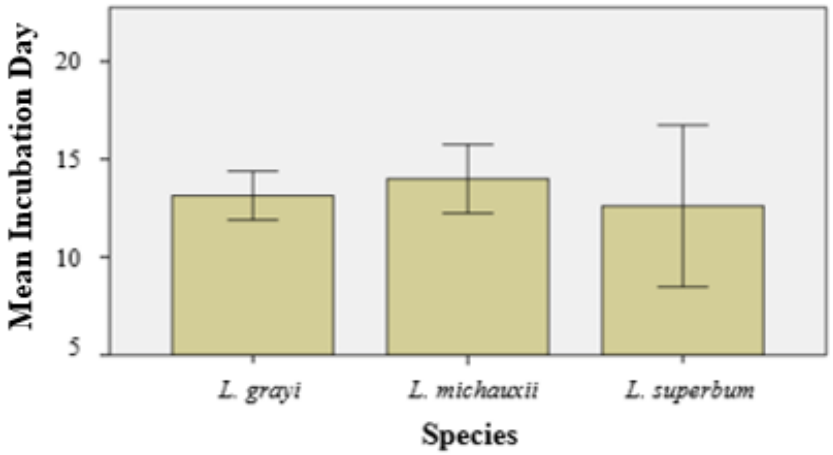


Figure 26. Comparison of incubation time (in days) for all three fungal lesion experiments by species–*L. grayi*, *L. michauxii*, and *L. superbum*. Bars represent 95% confidence interval (CI).

Fungal Inoculation Trials – Plant Survival

Plant survival post inoculation

Assumption Testing. To determine if a difference in plant survival was seen post infection with *P. inconspicua*, a two-way repeated measures ANOVA was conducted. There were no outliers identified (Figure 27). The data was normally distributed, as determined by the Shapiro-Wilk test ($p = 0.12$). The assumption of homogeneity of variance was not violated, as determined by the Levene's test ($p = 0.38$) (Lund 2015).

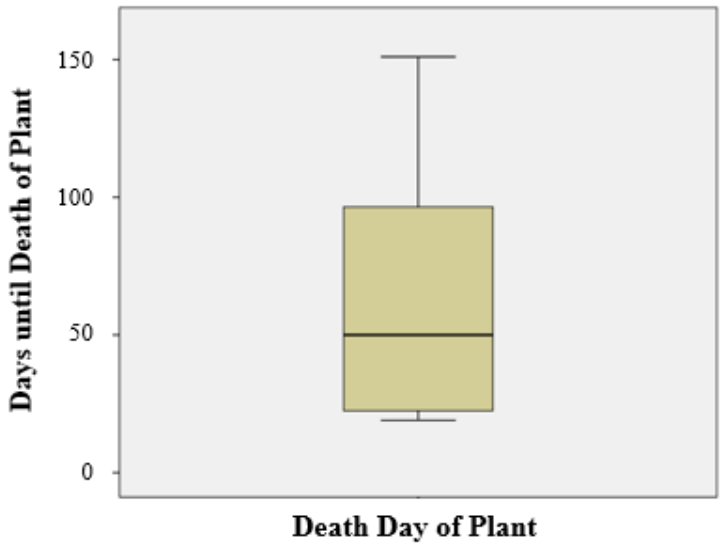


Figure 27. Plant survival days post inoculation for all three fungal lesion experiments. Bars extending below and above the box represent the range of incubation time. The heavy line represents the median. The extent of the box above and below the heavy line represents the 75th and 25th percentiles, respectively. No outliers identified

Tests Results. The mean days a plant survived post inoculation did not differ significantly among inoculum types or among species (Tables 29; Table 30; Figure 28; Figure 29).

Table 29. Descriptive statistics showing mean days a plant survived post inoculation by inoculum type, species, and by interaction of inoculum and species. Inoculum types: 1 = *P. inconspicua* hyphae from *L. grayi*; 2 = *P. inconspicua* conidia from *L. superbum*. SD = standard deviation and N = sample size.

	Factors	**Mean survival of plant (days)**	**N**
Inoculum	Hyphal culture from *L. grayi*	44	5
	Conidia from *L. superbum*	62	3
Species	*L. grayi*	23	2
	L. michauxii	71	2
	L. superbum	65	4
Interactions	1**L. grayi*	19	1
	1**L. michauxii*	19	1
	1**L. superbum*	95	3
	2**L. grayi*	26	1
	2**L. michauxii*	123	1
	2**L. superbum*	36	1

Table 30. Summary of two-way ANOVAs for plant survival post inoculation by inoculum type, species, and by interaction of inoculum and species. No significant p-values were found.

	DF	**Test statistic**	**P**
Inoculum	1	0.22	0.62
Species	2	0.63	0.69
Inoculum*species	2	1.67	0.37

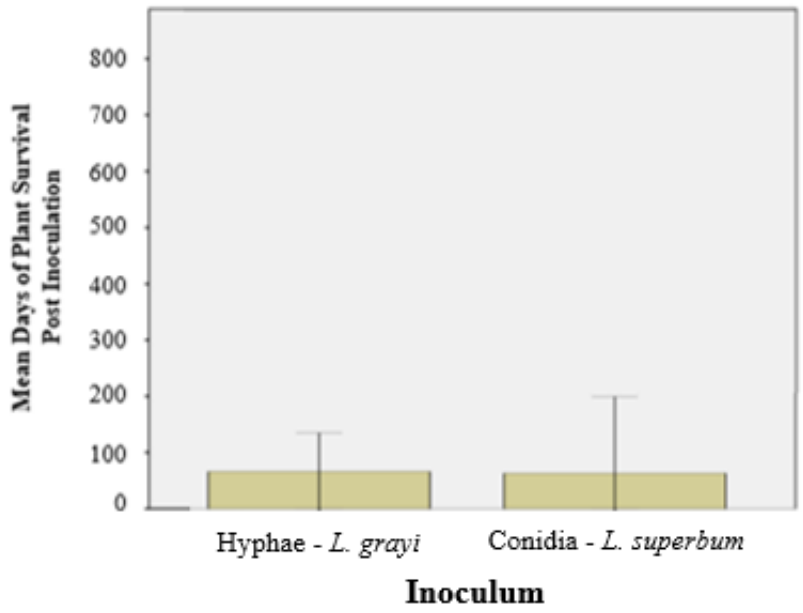


Figure 28. Comparison of experimental plant survival days post inoculation for all three fungal lesion experiments by *P. inconspicua* inoculum type–*P. inconspicua* hyphae from *L. grayi* and *P. inconspicua* conidia from *L. superbum*. Bars represent 95% confidence interval (CI).

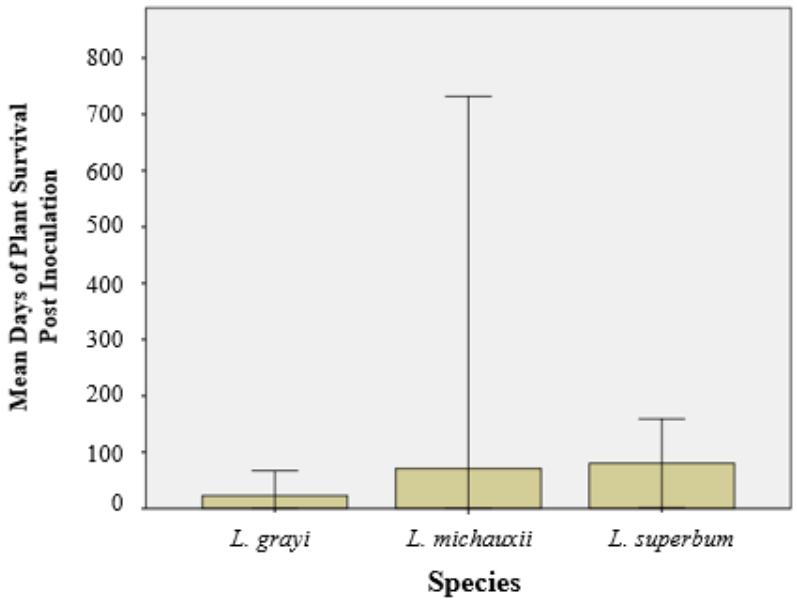


Figure 29. Comparison of mean experimental plant survival days post inoculation for all three fungal lesion experiments by species–*L. grayi*, *L. michauxii*, and *L. superbum*. Bars represent 95% confidence interval (CI).

Fungal Inoculation Trials – Death of Experimental Leaf

Death of experimental leaf. Abrasions without lesion development excluded

Assumption Testing. To determine if there was a difference in number of days until the experimental leaf senescenced from disease, a two-way ANOVA was performed. There were no outliers identified (Figure 30). The data were not normally distributed, as determined by the Shapiro-Wilk test ($p = 0.03$). The assumption of homogeneity of variance was violated, as determined by the Levene's test ($p = 0.01$) (Lund 2015).

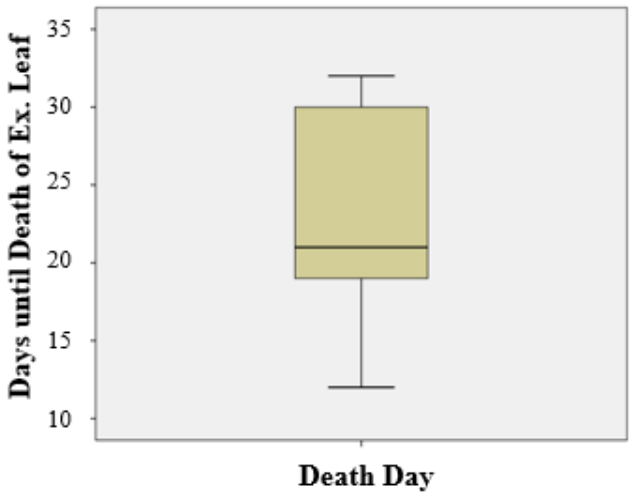


Figure 30. Survival days of experimental leaf post inoculation for all three fungal lesion experiments. Bars extending below and above the box represent the range of incubation time. The heavy line represents the median. The extent of the box above and below the heavy line represents the 75th and 25th percentiles, respectively. No outliers identified.

Test Results. No statistical difference was seen in inoculum type or species regarding the number of days it took for an experimental leaf to die (Table 31; Table 32; Figure 31; Figure 32). A visual determination of death was made.

Table 31. Descriptive statistics of mean days until experimental leaf died regarding inoculum type, species, and by interaction of inoculum and species, where 1 = *P. inconspicua* inoculum hyphae from *L. grayi* and 2 = *P. inconspicua* conidia from *L. superbum*. Standard deviation denoted by SD and sample size by N.

	Factors	**Mean days until leaf died**	**N**
Inoculum	Hyphal culture from *L. grayi*	20	9
	Conidia from *L. superbum*	25	7
Species	*L. grayi*	3	4
	L. michauxii	3	3
	L. superbum	2	9
Interaction	1**L. grayi*	21	2
	1**L. michauxii*	32	1
	1**L. superbum*	21	6
	2**L. grayi*	19	2
	2**L. michauxii*	28	2
	2**L. superbum*	24	3

Table 32. Summary of two-way ANOVA for number of days until death of experimental leaf by inoculum type, species, and by interaction of inoculum and species. No significant p-values were found.

Source	**DF**	**Test statistic**	**P**
Inoculum	1	1.86	0.20
Species	2	1.41	0.29
Inoculum*species	2	2.63	0.12

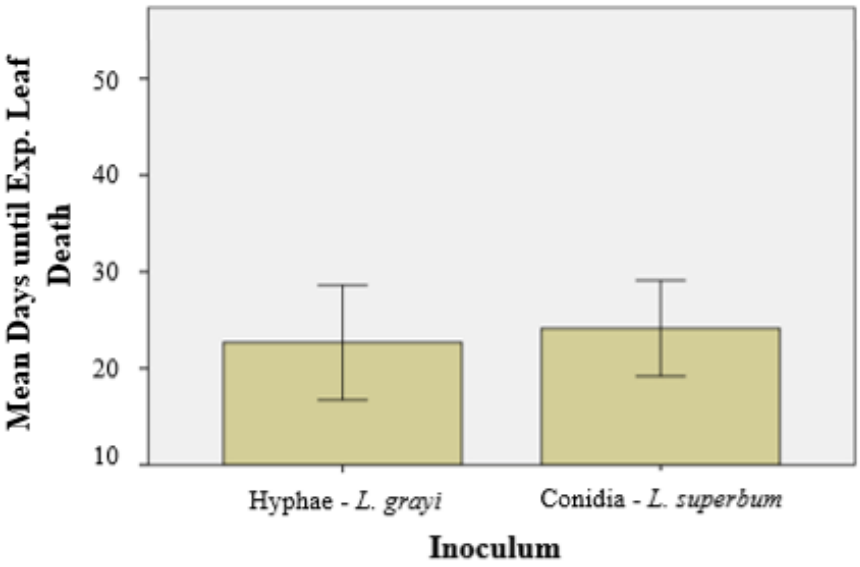


Figure 31. Comparison of mean experimental leaf survival days post inoculation for all three fungal lesion experiments by *P. inconspicua* inoculum type–*P. inconspicua* hyphae from *L. grayi* and *P. inconspicua* conidia from *L. superbum*. Bars represent 95% confidence interval (CI).

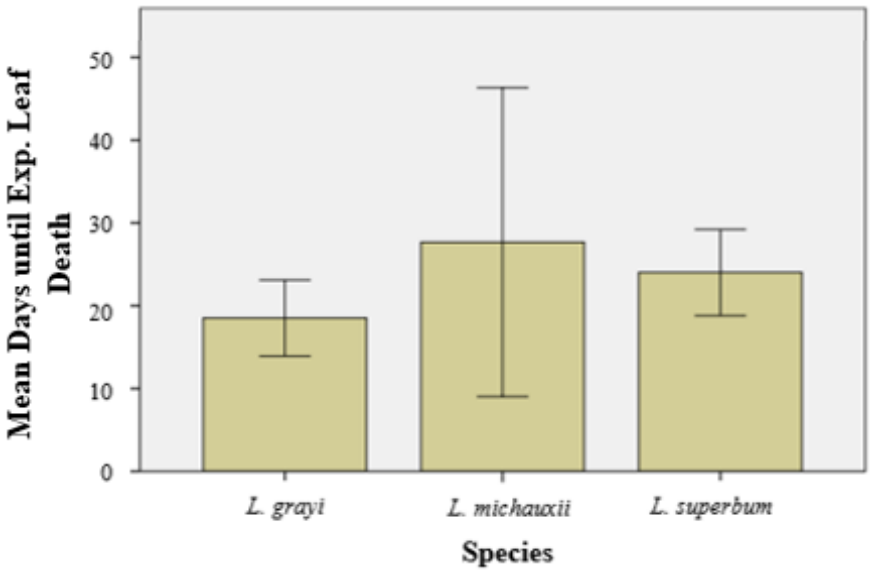


Figure 32. Comparison of mean experimental leaf survival days post inoculation for all three fungal lesion experiments by species–*L. grayi*, *L. michauxii*, and *L. superbum*. Bars represent 95% confidence interval (CI).

CHAPTER 4

DISCUSSION

Disease Extent

The current project has shown the disease was present throughout the range of *L. grayi* and ubiquitous within populations. Sample sites included: Bluff Mountain, Roan Mountain massif (Big Yellow Mountain, Rhododendron Gardens, Cloudland Hotel, Grassy Ridge), Whitetop Mountain, and four sites along the Blue Ridge Parkway). Disease was also present in all sampled *L. superbum* populations (Big Yellow Mountain, Bluff Mountain, Holston Mountain, Roan Mountain State Park, Unaka Mountain, Whitetop Mountain, and the Blue Ridge Parkway sites).

Variables Associated with Health

Several variables were identified in this study that were associated with lily health. Taller plants tended to be healthier than shorter plants in both *L. grayi* and *L. superbum* (Table 13; Table 14). Since *P. inconspicua* lesions take up leaf area and cause foliar damage, thereby disrupting photosynthesis, plants with a more leaves would have a greater leaf surface area and presumably, an advantage against the pathogen. Taller plants would also have an advantage in escaping inoculum as rain splash is a known vehicle for disease transmission (Madden 1997) and it would spread *P. inconspicua* inoculum from infected leaf litter to lower leaves. If fungal entry occurs through leaves, then smaller plants and lower lying leaves would be the most susceptible, as they experience a higher inoculation rate and a greater chance of contracting fungal infection.

In this study, the mean height of *L. grayi* exceeded that of *L. superbum* (Table 15). However, Skinner states that *L. superbum* is the taller species (2002). The discrepancy could lie

in an intentional survey bias. In the majority of sample sites, *L. grayi* was mixed with other *Lilium* species. Vegetatively, *L. grayi* looks nearly identical to other *Lilium* species state (Table 1). To maintain data integrity, only plants for which a definitive identification was possible, or previously marked plants of *L. grayi* were censused (Table 3 and 4). Since flowering individuals are taller than juveniles, this sampling bias would invariable skew the average height. In contrast, the *L. superbum* populations censused were known pure populations (Table 3 and 4), which allowed for inclusion of smaller, nonflowering individuals to be censused.

In *L. grayi*, plants in bald habitats and in full sun tended to be healthier than those in lower light conditions and other habitats. The sun effect may be caused by greater air flow and more light, both of which reduce humidity. Lower humidity, in turn, lowers fungal spore germination and viability (Block 1953), conditions that would ultimately cause less disease. This result appears to conflict with previous work, which did not identify any morphologic predictors of health for *L. grayi* (Ingram 2013). However, that study monitored only *L. grayi* on Roan Mountain, a site with a relatively uniform habitat and light condition. The current study included a wider range of habitats and light conditions, which facilitated a more comprehensive analysis of predictors of health. The Roan Mountain bald habitat was also the only population in full sun in the present study, so more study is needed to determine which factor, light or habitat, is more closely associated with health.

Diseased Populations and Individuals

Lilium superbum was first investigated as a host species of *P. inconspicua* because observations of *L. superbum* populations revealed disease symptoms similar to those in infected populations of *L. grayi*. Both species exhibited mass infection, chlorosis, wilting, and early senescence. At an individual level, both species had tan fungal lesions that when sampled,

yielded *P. inconspicua* conidia (Figure 34; Figure 35; Figure 36). The two species differ at the individual level. *Lilium superbum* lesions are slightly more linear than the circular *L. grayi* fungal lesions. There were also more *L. superbum* survivors later in the season than in *L. grayi*. Differing pattern of survivors were also seen. *Lilium grayi* health is clustered (Figure 33) and *L. superbum* has sporadic healthy individuals. This suggests *L. grayi* survivorship is due to environmental factors and *L. superbum* might be less susceptible to the disease.

Figure 33. *Lilium grayi* individuals, on left typical observation from population sites (August). On right, clustered late season survivors of *L. grayi* on Roan Mountain (September).

Figure 34. Similar population observations of a *L. grayi* (top) and *L. superbum* (bottom). Photographs taken in early summer.

Figure 35. Similar observations of *L. gray* (left) and *L. superbum* (right) individuals. Photographs taken in May for *L. grayi* and July for *L. superbum*.

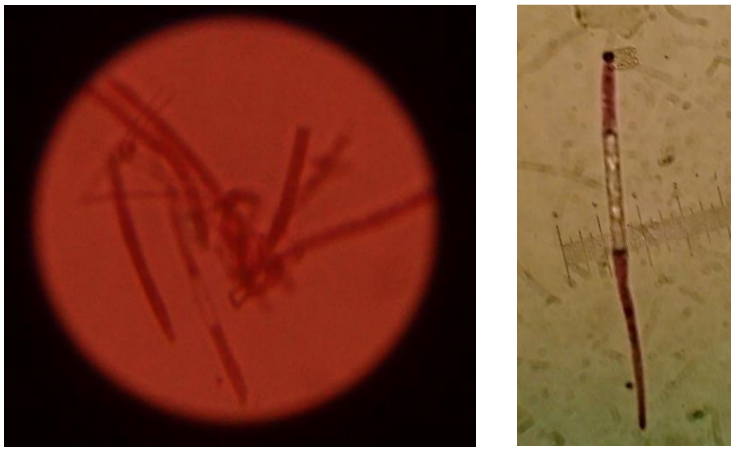


Figure 36. *Pseudocercosporella inconspicua* conidia from lesion samples of a *L. grayi* (left) and *L. superbum* (right). Photographs taken at varying levels of magnification.

Disease Effect

The second health score was significantly lower than the first health score taken earlier in the growing season. In fact, it was common on the second survey to find only bare stems of lily plants because entire subpopulations had experienced early senescence (Figure 37). The magnitude of the decrease in the mean health score indicates disease has weakened plants, and in many cases, this caused the plant to senescence three to four months prematurely. Further study is needed to determine if this trend is sustainable for *L. grayi* and *L. superbum* or if reserve storage in bulbs is sufficient to maintain plants.

Figure 37. Photograph of 18 previously healthy *L. superbum* plants. Typical second census observation for both *L. grayi* and *L. superbum* in August and September.

Lilium grayi Reproduction

The number of capsules produced (N = 9) was significantly lower than the number of flowers (N = 55) recorded earlier in the season among the BRP1, Elk Hollow Preserve, Roan Mountain (Cloudland Hotel Site), and Whitetop populations (Figure 15). It is unknown at present the number of capsules needed to sustain a healthy population, as *L. grayi* is capable of asexual cloning. However, it is concerning that BRP1 and Whitetop did not secure any capsules during 2016.

Lilium superbum Reproduction

Time constraints prevented observations of reproduction of *L. superbum* to be made. However, it is suspected that a similar pattern of low seed capsule production is present in *L. superbum*. For example, a *L. superbum* population in Roan Mountain State Park produced no capsules in 2015 or 2016. The number of capsules needed to sustain a vigorous, healthy population for either species is not known.

Lilium canadense: A Disease-free Population

Analysis of 28 *L. canadense* plants at a population in the TNC Shady Valley Schoolyard Springs Preserve showed no infection throughout the 2016 season (Table 4, Figure 1). In stark contrast, disease was found at all other sample sites including nine *L. grayi* and three *L. superbum* populations (Table 3; Table 4). In epidemiology, the presence or absence of disease depends on three factors: the host, environment, and disease (Gordis 2014; Figure 38). One of these factors must be different at the Shady Valley site compared to all other sites. The host factor can be excluded, as *L. canadense* is a known host for *P. inconspicua* (Braun 1995), unless this population represents a previously unknown resistant population. Late season survivors are pictured in Figure 39. The environment of Shady Valley is like that of Big Yellow Mountain

where disease is present (Figure 40) except the latter is at high elevation and the former is at a low elevation. Both sites have a thick, herbaceous groundcover that often surround lily plants with the crowding and high humidity conditions which would be conducive to fungal infection. Therefore, environmental differences are an unlikely explanation for the health of the Shady Valley site. A more likely explanation may lie in the disease factor. Possibly, *P. inconspicua* conidia are not present at this site. Thus, site and vector isolation could explain the absence of disease. TNC Shady Valley Schoolyard Springs Preserve is located in a maintained field apart from infected sympatric *Lilium* populations. This location also receives fewer visitors who can unknowingly transfer the microscopic conidia from one plant to another. To test this hypothesis, potted *L. grayi* could be brought in for one season to see if the plants becomes infected, but they should not be allowed to set seed as interspecific hybridization could occur. The finding of this disease-free site is a significant anomaly, as further study could uncover factors underlying the healthy *L. canadense* population. Most importantly, if a factor is identified, this could aid in treatment of infected *L. grayi* populations. *Lilium michauxii* also was disease-free but for a different reason as discussed below in "Fungal inoculation: Two new hosts."

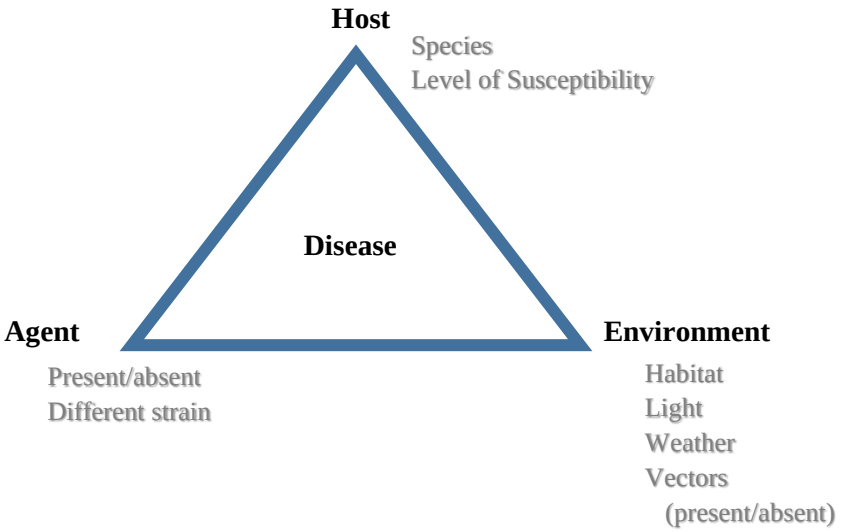


Figure 38. The three contributing factors of a diseased state: host, environment, and agent. Figure modeled after Gordis (2014).

Figure 39. Late season survivors of *L. grayi* (2Sep2016) and *L. canadense* (9Aug2016), both are known hosts for *P. inconspicua*.

Figure 40. Environment of healthy *L. canadense* population at Shady Valley, TN (top) and diseased *L. grayi* and *L. superbum* population on Big Yellow Mountain, NC (bottom).

Outbreak or Epidemic?

An epidemic and outbreak are similar in their definition as an "increase in the number of cases of a disease above what is expected" (Gordis 2014). However, an outbreak differs from an epidemic in several aspects. Most importantly, an outbreak is localized compared to a widespread epidemic ("Lesson" 2012). At all locations of *L. grayi* and *L. superbum*, *P. inconspicua* leaf spot disease was ubiquitous. These sample sites together represent an extensive range with occurrences in 11 counties and 3 states comprising the entire range of *L grayi* (Table 3; Table 4). *Pseudocercosporella inconspicua* and lily leaf spot disease therefore, meet the criteria of an extensive geographic occurrence that is essential for a disease to be characterized as an epidemic.

An outbreak is limited in magnitude. Although baseline data is not available for lily leaf spot disease, trends seen on Roan of up to 59% and of early senescence rates in 2013 (Ingram 2013) and up to 100% early senescence seen in this study should suffice as epidemic proportions.

An outbreak is transient not sustained over time. The effects of *P. inconspicua* on *L. grayi* have been noted for several decades, with several researchers noting fungal disease prior to a correct identification of the causal pathogen (Bates 1998; Donaldson 2003; Powell 2011; Ingram 2013; and Ulrey pers. comm.). This study also confirmed a sustained disease state for *L. grayi* for two consecutive years (Figure 13). These studies together provide sufficient evidence for a sustained disease state that is necessary for a disease to be considered an epidemic.

Additionally, characteristics of lily leaf spot disease aid, if not ensure, the fungal pathogen is widely dispersed and leads to an epidemic. It has a polycyclic conidia lifecycle, resilient, overwintering ascospores, and the fungal infection is not systemic. Together, this set of characteristics allow susceptible individuals to continually face the disease all season long and

return the following year to remerge through a pre-inoculated area. As a result, the epidemic can be expected to recur with similar amplitude year after year. High morbidity and extensive geographic occurrence support the hypothesis of *P. inconspicua* as an introduced species, as similar fungal epidemics with equivalent morbidity and range-wide extent were the result of a pathogen of exotic origin—examples include the chestnut blight of American chestnut and Dutch elm disease of North American elms (Schlarbaum 1998).

Inoculum Type or Host Source?

The fungal inoculation trials utilized *P. inconspicua* inoculum from two host sources: *P. inconspicua* from *L. grayi* and *P. inconspicua* from *L. superbum*. Including inoculum from two sources stemmed from visual observations and later demographic statistical analysis of presumed increased susceptibility of *L. grayi* to *P. inconspicua* infection as compared to *L. superbum* (Table 15). However, in the fungal inoculation trial, there was a significant interaction between the inoculum type used and the species inoculated (Table 17; Table 20). A result that suggests there is not a more virulent genotype. The hypothesis of a more virulent strain affecting *L. grayi* is unlikely because one inoculum did not elicit a greater more sever response in the *Lilium* species (Table 18). (A borderline, $p = 0.06$, result was found in time period 3 where *P. inconspicua* hyphal culture from a *L. grayi* produced a larger lesion area than *P. inconspicua* conidia culture from a *L. superbum*. See Table 18.) It should be noted that care was taken to gather hyphal inoculum from the perimeter of a fungal colony—the most actively growing area of the fungus—because hyphae may not always be infectious.

However, it is difficult to determine if inoculum type (hyphae or conidia) or host source of *P. inconspicua* inoculum (*L. grayi* or *L. superbum*) is responsible for the borderline result, $p = 0.06$, seen in lesion size that followed infection (Table 18). This is because only two types of

inoculum were available during the time experimental plants were viable—hyphal culture derived from an *L. grayi* individual and collected in the field from *L. superbum* lesions. Hence, the two factors are confounded. However, a plausible hypothesis for the differences points to inoculum type. The longer incubation period of the conidia inoculum would agree with the life cycle of a spore, where germination must first take place before infection proceeds. This lag time could explain why disease symptoms appear first when using hyphal culture because it skips the germination and early differentiation steps. Additional study is needed using inoculum that does not confound the two variables because an alternative hypothesis is that *P. inconspicua* isolates from one species is more virulent, i.e. has the shortest incubation period and elicits the largest lesion and chlorosis zone.

Leaf Lesions: Use of Chlorosis Measurements

Measurements and analysis of the chlorotic leaf area following inoculation were included because lesion measurements alone did not accurately represent the visual appearance of disease progression on leaves. For example, in many experimental leaves, the chlorosis would surpass the lesion and consume the entire leaf. Consequently, the leaf would wither and die, even though the inoculation lesion did not continue to expand. In other experimental leaves, the plant appeared to wall off the infection and much of the leaf remained green and apparently healthy. In both cases, the lesion size was similar. Therefore, measurements of lesion size and chlorosis size provided a more accurate quantitative representation of the differing disease states, progression, and severity.

Chlorotic Area Size

Although not specifically studied in *P. inconspicua*, other *Cercospora* species (sometimes a synonym of *Pseudocercosporella*) produce a toxin called cercosporin (Daub 2000). A cercosporin or a similar toxin would explain the chlorosis seen around and oftentimes far surpassing the *P. inconspicua* lesions. The chlorotic area data suggest a more widespread area of chlorosis on *L. grayi* compared to *L. superbum*. Possible explanations for the difference are that *P. inconspicua* produces more toxin in *L. grayi*, or if similar amounts are produced, *L. grayi* has a lower tolerance to the toxin or a more toxic version of the toxin is produced.

Direction of Chlorosis and Lesion Spread

Braun described circular *P. inconspicua* lesions on *L. grayi* and similar species (1995). Inoculations of *L. superbum* showed a more elliptic, nearly linear trend. Walling off of fungal lesion and chlorosis spread was seen in many experimental leaves (Figure 41), this trend was also seen in *L. grayi* and *L. michauxii* but to a lesser extent. As *Lilium* species have a vascular system with parallel veins, it is possible the fungal hyphae and toxic products are traveling along the vasculature and causing the somewhat linear lesions.

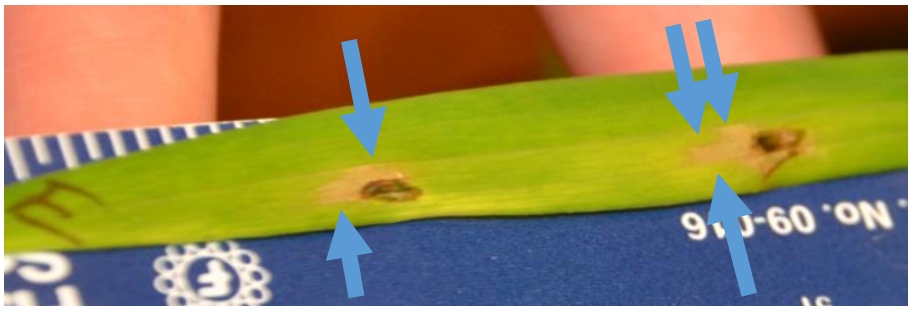


Figure 41. Observation of restricted lesion spread across parallel veins in *L. superbum*. This trend was noted in all species inoculated (*L. grayi*, *L. michauxii*, and *L. superbum*) but most frequently seen on *L. superbum*.

Toxin and Chlorosis

It would be most informative to determine if stem and leaf senescence was caused by a cercosporin-like toxin produced by *P. inconspicua*. *Lilium grayi* can develop extensive chlorosis before lesions develop (Figure 42). These observations combined with the prompt senescence suggest *L. grayi* are intolerant of a systemic factor such as a toxin. The response was greater than what appeared attributable to foliar damage of fungal lesions. If so, differences in the extent of chlorosis (when using the same inoculum type) suggest varying tolerances to such a toxin. Therefore, a working hypothesis is that *L. superbum*, with smaller chlorosis zones is perhaps more tolerant of the toxin.

Figure 42. Observation of systemic chlorosis that exceeds the lesion area in an inoculated abrasion. Individual pictured is a *L. grayi* thirteen days post inoculation.

Lesion Size

Varying host susceptibility is one explanation for the borderline result seen in lesion size (Table 19). Another explanation could lie in plant structure. Perhaps the cellular walls of the *L. grayi* venous system of *L. grayi* are more permeable, allowing the toxin to cross the parallel veins and have a more systemic effect than that observed in *L. superbum*. In support of this, observations of hindered lesion spread across the parallel veins was seen in all species, but the most pronounced and frequent restriction to intervein areas was in *L. superbum* (Figure 41).

Host Susceptibility

If the chlorotic area is an indicator of disease severity, then a significant differences in lesion size, chlorotic area, or both would suggest varying levels of host susceptibility among the three *Lilium* species.

However, the inoculation trials revealed there was not a difference in response to *P. inconspicua* inoculum between the three *Lilium* species. This result would not indicate a difference in host susceptibility. (A borderline, p=0.054, result was found in time period 4 where *L. michauxii* had a larger lesion area than *L. grayi*. See Table 19.)

This agrees with the disease observations in the field where *L. grayi* and *L. superbum* act similarly on a population level (Figure 34) and on an individual level (Figure 35).

Analysis Shortcomings

Increased sample size would increase the power of data analysis in the inoculum experiments. However, with concerns for the threatened plant status, it was difficult and not ecologically feasible to obtain more *L. grayi* plants. Plant transfer shock and complications of artificial overwintering were contributing factors in lower plant sample size of *L. michauxii* and

L. superbum. *Lilium canadense* was not included in the analysis because the unusual healthy population was not identified until the end of the second year of study.

Increased sample size would allow for abrasions to be placed on different leaves, and if enough plants were secured, only one leaf per plant would be inoculated. This would eliminate concerns for nesting and all observation would remain independent. Again, due to difficulty in obtaining plants, it was necessary to have two abrasions per experimental leaf and two experimental leaves per plant (provided the plant had sufficient leaf whorls).

Number of Days until Plant and Leaf Death

Although no significant difference was seen in mean survival days post inoculation (Table 30), several small and large lab inoculated plants of *L. superbum* individuals were capable of living with infection until the time when natural, seasonal senescence would be expected. See Figure 43. (One large *L. michauxii* also had long survivorship). More study is needed to determine if height is a contributing factor for longer survival following infection for *L. superbum* and *L. michauxii*. Survival of infected *L. grayi* was short (but still insignificant). No *L. grayi* survived longer than 26 days. This result is in agreement with observations seen in the field where populations and individuals of both species had diseased states that are nearly identical (Figure 34; Figure 35).

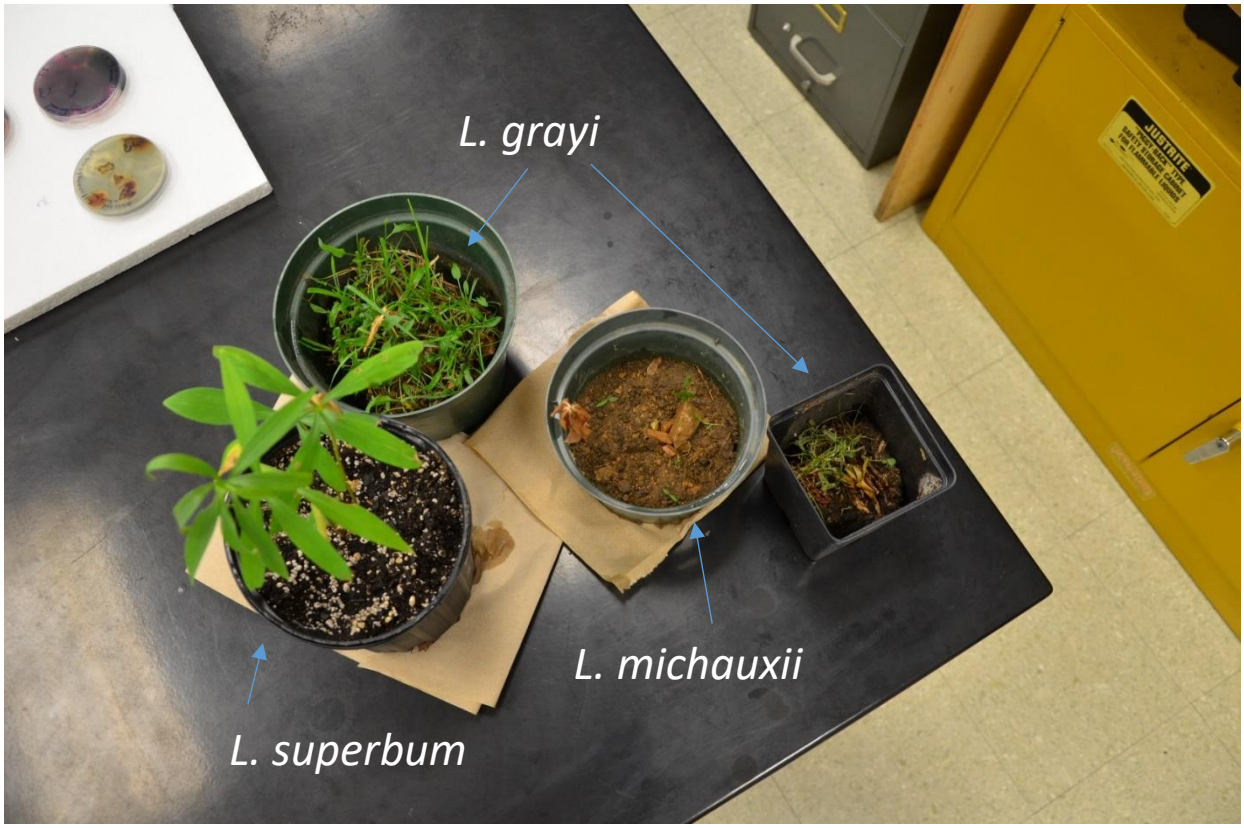


Figure 43. End of fungal inoculation trial two where both *L. grayi*, and the *L. michauxii* senesced under fungal pressure, but the *L. superbum* did not.

Fungal Inoculation: Two New Hosts

At the onset of the study, it was hypothesized that *L. superbum* was a host for *P. inconspicua* due to field observations and positive conidia recovery from samples. However, all observed *L. michauxii* individuals were healthy throughout the season (Table 2). Perhaps most intriguing is the observation of a browsed, healthy *L. michauxii* individual surrounded by unhealthy *L. superbum* plants stricken with *P. inconspicua* infection.

Inoculation of *L. michauxii* led to infection, a result that provides valuable insights into the underlying explanation and mechanism of its sustained health in the field in the presumed presence of disease inoculum. Occurrence of infection after leaf abrasion and after inoculation suggests that *L. michauxii* has mechanical resistance to *P. inconspicua* and that *L. michauxii's* thicker cuticle and preference for a dryer habitat offer this species an advantage to *P. inconspicua* infection in the field. The severity of the infection after inoculation makes the diagnosis of a hypersensitive response unlikely, which would have pointed to a general but not genetic immunity to disease (Mur et al. 2008). Since infection was inducible, it is also unlikely that genetic resistance is present in *L. michauxii*. This hypothesis suggests the development of a genetically resistant *L. grayi*—whether naturally, evolved, developed through breeding programs, or conferred through genetic manipulation—is much less promising.

Lilium michauxii usually grows in a drier environment than both *L. grayi* and *L. superbum*, giving it another possible advantage against fungal infection. Such an environment, with a presumed smaller spore load of *P. inconspicua*, would also decrease the chance of contracting infection if the cuticle were inadvertently breached after browsing or other damaging factors.

Infection followed inoculation of *L. superbum*, a finding that confirms the disease observations seen in the field (Figure 36) and further supports the host species hypothesis. Some underlying genetic resistance may be found in *L. superbum*. Lesions of *L. superbum* do not tend to cross leaf veins as frequently as lesions in *L. superbum* or *L. michauxii* (Figure 41; Figure 44).

Figure 44. Typical experimental leaf observation seen during the fungal inoculation trials. Photograph on left is a *L. grayi* experimental leaf, the middle is a *L. superbum*, and the right is a *L. michauxii*.

Reservoir Hypothesis

Pseudocercosporella inconspicua conidia and lily leaf spot disease were identified on *L. superbum* in the field and successful inoculation of *L. superbum* in the lab confirmed this species can act a host of *P. inconspicua*. A reservoir is defined as a host where disease can successfully survive, i.e. one that is "chronically infested with the causative agent of a disease and can act as a source of further infection" (Gordis 2014). My findings support the characterization of *L. superbum* as a reservoir given the extensive presence of *P. inconspicua* at every *L. superbum*

location sampled (Table 3), and sampling throughout the season yielded *P. inconspicua* conidia. Populations of *L. superbum* have an overlapping and lower elevation distribution than *L. grayi* (Skinner 2002). Having been confirmed as a host for *P. inconspicua*, being a host and having the ability to co-occur with *L. grayi*, *L. superbum* would become a source of disease, with infected leaves of previous years contaminating leaf litter and providing inoculum for next year's infection and the genesis for future fungal epidemics. Therefore, *L. superbum* should be regarded a reservoir of disease for *L. grayi*.

Although the fungal inoculation experiments identified *L. michauxii* as a host species, *L. michauxii* does not serve as a functional host in the field, as all censused individuals were healthy across the region and throughout the season and none carried *P. inconspicua* conidia (Table 2). Since *L. michauxii* does not succumb to, transfer, or harbor infection in the field, my findings do not support the characterization of *L. michauxii* as a reservoir.

The finding that *L. superbum* is a host for *P. inconspicua* and that *P. inconspicua* leaf spot disease is a concern in *L. superbum* presents more complex challenges for management of *L. grayi* populations. If a treatment for *P. inconspicua* is found, management of this additional disease reservoir would require more time and money to be allocated from an already strained fiscal budget of land and conservation agencies. Conversely, there is a benefit in the knowledge that *L. superbum* is a host for *P. inconspicua*. Being more common and growing near *L. grayi* populations in similar habitats, this species could be considered a surrogate for experimental and conservation regimens leaving the *L. grayi* and its fragile habitat undisturbed until an appropriate and effective approach to management of the fungal pathogen is found.

Future Study

Additional, more extensive sampling for *P. inconspicua* conidia should be performed to determine the full extent of the *P. inconspicua* host range. Mycological and herbarium occurrence data indicate *P. inconspicua* in five northern states (Maine, Michigan, New York, Vermont, and Wisconsin) and two Canadian provinces (Alberta and Manitoba) (Ingram et. al 2017). Since *L. canadense* and *L. superbum*—both hosts of *P. inconspicua*—have distributions that overlap these northern areas with the regional disease occurrences in Tennessee, North Carolina, and Virginia, it is a reasonable hypothesis that *P. inconspicua* has a continuous distribution from Canada to Georgia.

It would be interesting and informative to include cultivated lilies species in fungal inoculation experiments. Since Asiatic lilies have thicker cuticles, one would expect the results of fungal inoculation experiments to be similar to that of *L. michauxii,* where infection is inducible but not seen in the field. Species native to central and western North America should also be included in future studies, to help determine the point of origin of *P. inconspicua* to North America, as an introduction from across Pacific rather than the Atlantic is plausible. In fact, infection has already occurred in plant nurseries in Russian and Japanese plant nurseries (Makoto 1925; Zerova 1940). Similar infections would have substantial economic consequences for the nursery industry in North America. The introduction of invasive fungal infections such as the chestnut blight and pine blister rust was, in part, the impetus for the US Plant Quarantine Act of 1912 (Palm 2001).

To further strengthen future inoculation trials, a plant which cannot be infected with *P. inconspicua* (a negative control for the inoculum) should be added. This would allow additional safeguards on the viability and restriction of the inoculum. A negative result in this control plant

and a positive result in a known host species would help prove that the inoculation was successful.

A genetic analysis would also be a necessary next step for untangling the historical occurrence and native status of *P. inconspicua* in North America. Genetic samples were taken in this study while gathering fungal inoculum and recording demographic data. Isolation of *P. inconspicua* from these samples has proven successful for many populations.

Concerns for Species Viability

Several factors are present that together raise concern for the viability of *L. grayi* as a species. First, as a narrow, southern Appalachian endemic with a preference for high elevation sites, this species has a restricted geographic and ecological distribution. Second, *L. grayi* has complex hypogeal seed germination. Deno (1993) completed an extensive germination study where *L. canadense* var. *editorum*—a species closely related to *L. grayi*—was studied. Deno speculated the germination rates of this species would closely resemble that of *L. grayi*. Laboratory treatment of fresh seeds following a shifting temperature protocol had a 94–96% germination rate. However, field experiments using fresh seeds started in September—that experienced the warm and cool treatment needed for hypogeal germination—had 0% germination (Deno 1993). Third, if germination was obtained, it takes four to five years for a seedling to reach reproductive maturity. Fourth, high morbidity and mortality associated with *P. inconspicua* add additional stress to this species. Disease has also been shown to decrease seed viability and capsule production (Ingram 2013). Fifth, *L. grayi* can occur in mixed populations with other species such as *L. canadense* and *L. superbum* where this causes undesirable competition for space, nutrients, and light. Decline due to exotic plant competition has been noted by other researchers working with endemic plants (Gioria & Osborne 2014). Competition

with taller plants limits light—a necessary element for flowering and general health (Deno 1993). If seedlings do not receive adequate light, they can remain suspended in the single leaf stage for years (Deno 1993). Lastly, the situation becomes worse when the graceful but conspicuous blooms make *L. grayi* a target for illegal collection (Dunscomb 2009) and their threatened status makes them all the more attractive to collect.

Through this research lily leaf spot disease and it causal pathogen, *P. inconspicua*, were present at every location sampled and was the likely cause of declining health seen throughout the entire *L. grayi* range. *Lilium michauxii* and *L. superbum* have been identified as new hosts for *P. inconspicua* and *L. superbum* is characterized as a reservoir of disease, serving as an additional threat to the viability of *L. grayi* populations. The epidemic nature of *P. inconspicua* and the high morbidity and mortality it fosters create a disease trend that, even for an r-selected species, is concerning. Given (i) the susceptibility of *L. grayi* demonstrated in both the demographic health analysis and inoculation experiments, (ii) considering the historically low occurrence of *P. inconspicua* in North East America (Ingram et al 2017), and (iii) the range-wide occurrence of *P. inconspicua* among the *L. grayi* species, one could postulate that *L. grayi* shows a response to the *P. inconspicua* pathogen typical of a naïve host newly exposed to an exotic pathogen. In addition to disease, *L. grayi* species must overcome several serious threats including habitat loss, complex seed germination, competition, browsing, and poaching. Therefore, the lily leaf spot disease is not only a threat in itself, but by weakening plants it also exacerbates the other threats. It is not one single factor but a combination of the threats that ultimately endangers *L. grayi*.

REFERENCES

Bates M. 1998. Monitoring Protocol for *Lilium grayi*, pp. 1-7. North Carolina Plant Conservation Program. Available by request from: U.S. Fish and Wildlife Service.

Block SS. 1953. Humidity requirements for mold growth. Applied microbiology, 1(6):287. [Internet]. [Cited 2 Feb 2017]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1056928/pdf/applmicro00290-0019.pdf

Braun U. 1988. Studies on *Ramularia* and allied genera (II). Nova Hedwigia 47:335-349.

Braun U. 1995. A monograph of *Cercosporella, Ramularia*, and allied genera (Phytopathogenic Hyphomycetes) 1:147.

Deno NL. 1993. Seed germination: Theory and practice (2nd ed.). [Internet]. [Cited 20 June 2016]. Available from: https://naldc.nal.usda.gov/download/41278/PDF

Daub ME, Ehrenshaft M. 2000. The photoactivated cercospora toxin cercosporin: Contributions to plant disease and fundamental biology. Annual Review of Phytopathology. 38:461-490.

Donaldson J. 2003. Big Yellow Mountain *Lilium grayi* monitoring, pp. 1-44. Contracted unpublished report submitted to The Nature Conservancy (contract # NCFO-021403-01).

Dunscomb. 2009. "Results detailed report." *Lilium grayi*. NatureServe. [Internet]. [Cited 17 Jun 2016]. Available from:

http://explorer.natureserve.org/servlet/NatureServe?searchSciOrCommonName=Lilium+Grayi&x=0&y=0

Finnegan JT, Robinson LG. 2014. Natural Heritage Program list of rare plant species of North Carolina, p. 39. North Carolina Natural Heritage Program, Office of Land and Water Stewardship, N.C. Department of Environment and Natural Resources, Raleigh, NC. [Internet]. [Cited 03 Jun 2016]. Available from: http://dncr-nhp.s3.amazonaws.com/s3fs-public/documents/files/2016-nhp-list-of-rare-plants-of-nc-revised-02-24-2017.pdf

Fletcher J, Luster D, Bostock R, Burans J, Cardwell K, Gottwald T, Smith K. 2010. Emerging infectious plant diseases. Emerging Infections. 9:337-366. [Internet]. [Cited 10 Jun 2016]. Available from: http://swfrec.ifas.ufl.edu/hlb/database/pdf/0_Fletcher_10.pdf

Ellison AM, Bank MS, Clinton BD, Colburn EA, Elliott K, Ford CR, Mohan J. 2005. Loss of foundation species: consequences for the structure and dynamics of forested ecosystems. Frontiers in Ecology and the Environment. 39:479-486.

Gioria M, Osborne BA. 2014. Resource competition in plant invasions: Emerging patterns and research needs. Frontiers in plant science. 5:501. [Internet]. [Cited 20 Jun 2016]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4179379/

Gordis L. 2014. *Epidemiology* (Fifth edition). Philadelphia: Elsevier Saunders.

Hertsenberg A, Noori M. 2010. Plate Streaking & Growth. [Internet]. [Cited 5 July 2016]. Available from: http://fg.cns.utexas.edu/fg/protocol:_plate_streaking_and_growth.html

Ingram RJ. 2013. Cause and impacts of the early season collapse of *Lilium grayi* (Gray's lily), on Roan Mountain, TN/NC. MS Thesis Biology, East Tennessee State University,

Johnson City, TN. Electronic Thesis and Dissertations. Paper 1190. [Internet]. [Cited 5 Aug 2015]. Available from: http://dc.etsu.edu/etd/1190/

Ingram R. 2015. Ph.D. Candidate, Dept. of Plant Pathology, Univ. of GA.

Ingram R, Levy F, Barrett C, Donaldson J. 2017. Mining herbaria for clues to the historic prevalence of lily leaf spot disease (*Pseudocercosporella inconspicua*) on Gray's Lily (*Lilium grayi*) and Canada Lily (*L. canadense*). *Rhodora* (in press).

Lesson 1: Introduction to Epidemiology. 2012. Centers for Disease Control and Prevention. [Internet]. [Cited 21 Jan 2017]. Available from: https://www.cdc.gov/ophss/csels/dsepd/ss1978/lesson1/section11.html

Lund A. 2015. Two-way repeated measures ANOVA: Assumptions. [Internet]. [Cited 21 Jan 2017]. Available from: https://statistics.laerd.com/premium/spss/twrma/two-way-repeated-measures-anova-in-spss-8.php

Mack RN, Simberloff D, Mark W, Evans H, Clout M, Bazzaz FA. 2000. Biotic invasions: causes, epidemiology, global consequences, and control. Ecological Applications, 10. 3:689-710.

Madden LV. 1997. Effects of rain on splash dispersal of fungal pathogens. Canadian Journal of Plant Pathology. 19(2):225-230.

Makoto H. 1925. On a Cercosporellose of the cultivated lily, [in Japanese]. Ann. Phytopathol. Soc. Japan 1:20-30. [Internet]. [Cited 20 Jan 2017]. Available from: https://www.jstage.jst.go.jp/article/jjphytopath1918/1/6/1_6_20/_article

Mur LA, Kenton P, Lloyd AJ, Ougham H, Prats E. 2008. The hypersensitive response; the centenary is upon us but how much do we know? Journal of experimental

Botany. 59(3):501-520. [Internet]. [Cited 2 Feb 2017]. Available from: https://www.ncbi.nlm.nih.gov/pubmed/18079135

Palm ME. 2001. Systematics and the impact of invasive fungi on agriculture in the United States: Knowledge of the systematics of plant-inhibiting fungi is fundamental for making appropriate plant quarantine decisions and thereby safeguarding US plant resources. *BioScience*. 51:141-147. [Internet]. [Cited 1 Jul 2016]. Available from: https://academic.oup.com/bioscience/article/51/2/141/393410/Systematics-and-the-Impact-of-Invasive-Fungi-on

Powell J. 2011. Impacts of goat browsing and disease on *Lilium grayi*, Gray's Lily, on Roan Mountain. Undergraduate Honors Thesis Series. East Tennessee State University, Johnson City, TN. Paper 16. [Internet]. [Cited 20 Aug 2015]. Available from: http://dc.etsu.edu/honors/16/

Radford A, Ahles H, Bell C. 1968. *Lilium* L., In, Manual of the Vascular Flora of the Carolinas, pp. 309-311. University of North Carolina Press, Chapel Hill.

Schlarbaum SE, Hebard F, Spaine PC, Kamalay JC. 1998. Three American tragedies: chestnut blight, butternut canker, and Dutch elm disease. [Internet]. [Cited 2 Feb 2017]. Available from: https://www.nrs.fs.fed.us/pubs/jrnl/1998/1998_exotic-pests-papers/schlarbaum_1998-exoticpests.pdf

Schnepf R. 2005. Asian soybean rust: Background and issues. In CRS Report for Congress Vol. 12. [Internet]. [Cited 22 Sep 2015]. Available from: http://nationalaglawcenter.org/wp-content/uploads/assets/crs/RL32225.pdf

Skinner MW. 2002. Flora of North America, *Lilium grayi*. [Internet]. [Cited 25 Sep 2015]. Available from: http://www.efloras.org/florataxon.aspx?flora_id=1&taxon_id=242101731

Trigiano R, Windham M, Windham A. 2008. Plant Pathology: Concepts and Laboratory Exercises, pp. 143-150. CRC Press, Boca Raton, FL.

Ulrey C. National Park Service, Blue Ridge Parkway, Asheville, NC.

Weakley AS. 2012. Flora of the Southern and Mid-Atlantic States. UNC Herbarium, North Carolina Botanical Garden. [Internet]. [Cited 15 Jan 2017]. Available from: https://www.researchgate.net/publication/256065746_Weakley_AS_2012_Flora_of_th Southern_and_Mid-Atlantic_States_UNC_Herbarium_North_Carolina_Botanical_Garden_1225_pp

Zerova Mme MY. 1940. Diseases of lilies in Kieff nurseries [in Russian]. Botanichnii Zhurna 1: 143-147. [Internet]. [Cited 25 Jan 2017]. Available from: https://scholar.google.com/scholar?hl=en&q=Diseases+of+lilies+in+Kieff+nurseries&btnG=&as_sdt=1%2C43&as_sdtp=

VITA

CINDY BARRETT

Education: M.S. Biology, East Tennessee State University, Johnson City, Tennessee 2017

Research: Epidemiology and population impacts of a fungal disease of a rare lily. Coursework emphasis in Epidemiology and Statistics including: Epidemiology I, Genetic Epidemiology, Infectious Disease Epidemiology, Biometry, and Modeling Biological Systems. Current GPA: 3.88.

B. A. Biology, Tusculum College, Greeneville, Tennessee 2011

Extensive coursework in biology including: Microbiology, Genetics, Parasitology, Immunology, Human Physiology, and Global Environmental and Health Issues.

Graduated Magna cum Laude with a 3.94 GPA.

B.A. Art and Design, Studio Concentration, Tusculum College, Greeneville, Tennessee 2011

Focus on integrating art with biology to communicate abstract concepts and demonstrate complex patterns.

Presentations: ETSU Department of Biology Seminar 2015.

Roan Stewardship Committee 2015.

Roan Stewardship Committee 2017 - presentation and management suggestions.

ETSU Department of Biology Seminar 2016.

Friends of Roan Mountain Winter Rally February, 2017.

Publication: Ingram, R., Levy F., Barrett C., Donaldson J. 2017. Mining Herbaria for Clues to the Historic Prevalence of Lily Leaf Spot Disease (*Pseudocercosporella inconspicua*) on Gray's Lily (*Lilium grayi)* and Canada Lily (*L. canadense*). *Rhodora* (in press).

Awards: Marcia Davis Research Award -$500.

Friends of Roan Mountain Research Grant - $800.

Volunteer Experience: Caregiver for Father 2006–2007. Experiences with infectious disease (MRSA, *Clostridium difficile*, *Staphylococcus aureus*, and *Candida albicans*).

Caregiver for Grandmother 2012–2013. Experiences with noninfectious disease (Diabetes, Osteoporosis, and Dementia).

Algebra I tutor - Fall 2016 and Spring 2017