2015

Effect of canopy cover and specific leaf area on endophyte diversity in Rhododendron macrophyllum and Acer macrophyllum

Jesse Hughes
University of Puget Sound

Effect of canopy cover and specific leaf area on endophyte diversity in *Rhododendron macrophyllum* and *Acer macrophyllum*

Jesse Hughes and Brennan Schon

Abstract

Nearly all plants share an asymptomatic symbiosis with many different kinds of endophytes: fungi and bacteria that exist within the plants. Despite their pervasiveness, much remains a mystery surrounding why these relationships exist. It is known that in certain circumstances the endophytes provide pathogen resistance for host plants. The purpose of this study was to identify certain factors that affect endophyte diversity. We chose to measure specific leaf area and canopy cover while collecting leaves from *Rhododendron macrophyllum* and *Acer macrophyllum*. These were chosen to represent low and high SLA. We predicted that leaves with low SLA will have fewer morphospecies due to more investment in leaf structure, thus having more built-in defenses. Sections of these leaves were plated on a mycelium medium to watch for growth. Endophytes were morphotyped by size, shape, and color. As predicted, Maple leaves showed 23% greater endophyte richness than Rhododendrons. Assuming a beneficial symbiotic relationship, it might be the possible that certain plants with a greater investment in leaf structure are not as reliant on endophyte protection. Canopy cover was not shown to have a significant affect on endophyte diversity and it was the same across leaf-types. To further address this issue, more plants would be needed to get a broader spectrum of SLA values. Further studies should aim at identifying if the endophytes are providing their hosts benefits and what those might be.

Introduction:

Many symbiotic relationships exist within the natural world. Mycorrhizal fungi help plants improve phosphate ion acquisition due to the increased efficiency and mobility of mycelium vs. the plants' roots. In exchange for this support, the plants provide a constant source of nutrients like carbohydrates (Reece et al. 2011). These relationships are well studied and show that ecological trade-offs exist among different species. However, relationships among other fungi existing within the leaves are still in much need of elucidation. These fungal endophytes live asymptomatically within leaves and other parts of all plants. While many of the benefits are unknown, endophytes have shown to benefit certain plants by making them less susceptible to herbivores, increase tolerance to heat and drought and defend against pathogens (Arnold et al. 2003). Endophyte research is a particularly interesting field due in large part to the many

biological compounds which they produce. These compounds have great impact on the plant species and are becoming more important for their pharmaceutical applications (Aly et al. 2011).

Most endophytes are very specific for their host-species. However, this specificity can be influenced by macroclimate and microhabitat conditions (Aly et al. 2011). Endophyte diversity has been shown to be influenced by latitude (Higgins et al. 2007). These changes suggest that diversity of endophytes within the host plant can be shaped by physiological and geographical location. The goal of this research is to study the effect that specific leaf area (SLA), a functional leaf trait, has on endophyte diversity within the given host. SLA has been correlated positively with a plant's growth rate potential and maximum photosynthetic rate. Plants that have low SLA tend to have long life spans and invest heavily in leaf structural defenses (Cornelissen et al. 2003). As an additional factor, canopy cover will be measured to infer how light exposure can affect diversity. Particularly, these variables will be looked at in the leaves of *Rhododendron macrophyllum* and *Acer macrophyllum* (maple tree). These were chosen because of the obvious difference in specific leaf area and the corresponding assumption of a difference in energy investment. Based on previous studies showing the beneficial effects of endophyte symbiosis, we predict that leaves with lower SLA will have greater endophyte diversity because they will have less need for the added defense.

Methods

Collection

For this experiment 10 leaves each from *Rhododendron macrophyllum* and *Acer macrophyllum* were collected. During search in Point Defiance Park, trees were chosen randomly. Maple leaves were chosen that were still mostly green. Leaves with obvious pathogen or herbivory damage were avoided. Leaves were chosen haphazardly, one per plant/tree. Due to

the nature of maple leaves being much higher off the ground, height as a variable was not measured. During collection, a densiometer was used to measure canopy cover above each leaf, with an average from four compass readings.

Calculating SLA

Pictures were taken of each leaf and ImageJ software was used to measure area. 16mm^2 segments were cut from each leaf. The leaves were placed in a drying oven at 80°C for 48 hours and then weighed. Specific leaf area (SLA) = area (cm^2)/ dry weight mass (g). The following formula was used to correct dry leaf mass for the segment that was cut previous to drying:

$$\frac{Whole\ Area - 16mm^2}{measured\ dry\ mass} = \frac{Whole\ Area}{Pre-cut\ dry\ mass}$$

Plating Protocol

The 16mm^2 segments were sterilized in ethanol and further cut into 2mm^2 pieces. Each piece was placed on a 2% malt extract agar petri dish, with 8 rows and 8 columns. The plates were sealed and kept at room temperature in a dark drawer. After five days the plates were observed for growth and pictures were taken.

Morphotyping

Utilizing the plates and 5day growth pictures, plates were analyzed to determine number of endophyte species per plate. Each endophyte could be distinguished by color, texture, and shape.

Data Analysis

To analyze whether the data were statistically significant, a 3-factor ANCOVA was accomplished using R statistical computing software. SLA, leaf type and canopy cover were treated as explanatory variables and morphospecies number was the response variable. Due to SLA and leaf-type being confounding variables to the ANCOVA, independent t-tests were done

using leaf-type x morphospecies and SLA x leaf-type. A correlation test was done on SLA x number of morphospecies. All sampling variability assumptions were met.

Results

The effect that leaf type had on number of endophyte morphospecies was not dependent on SLA (ANCOVA, F=7.4, df=1,16, p=0.220; Figure 1). However, depending on which variable was placed first in R, either SLA or leaf type would be significant and the other variable would not. This is due to the fact that these variables are nearly interchangeable. This makes sense as the leaves were chosen based assumed SLA differences. Mean maple leaf SLA values were 807% greater (427.1 ± 32.95 (SE) cm^2/g) than rhododendron leaves (47.3 ± 3.55 (SE) cm^2/g; t-test, t = 11.46, df = 18, $p < 0.001$; Figure 2).

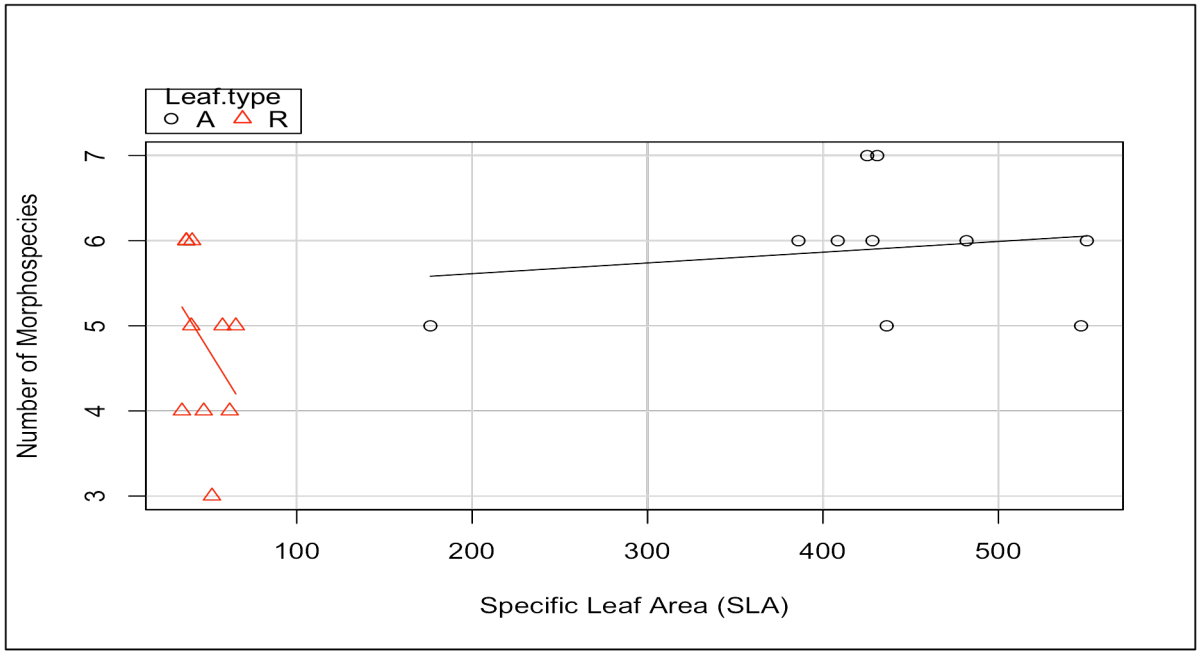


Figure 1. Effect of SLA and leaf-type on number of morphospecies (n=20 leaves). Ran ANCOVA twice with explanatory variables swapped in R. Both SLA (p=0.017) and leaf-type (p=0.015) showed a significant effect on morphospecies when placed first in the model and neither was dependent upon the other variable for that effect (p=0.220).

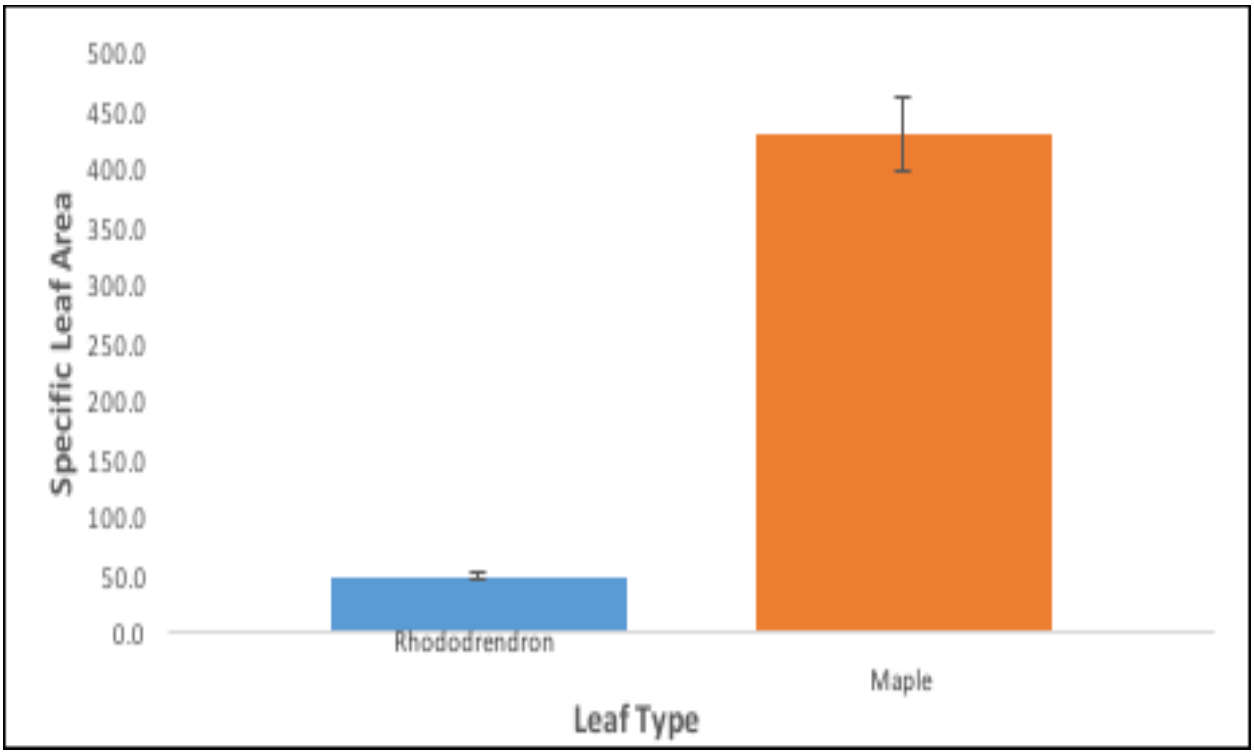


Figure 2. SLA values differed based on leaf type (±1 SE). Mean maple leaf SLA values (n=10 leaves) were 807% greater than rhododendron leaves (n=10 leaves, $p < 0.001$).

Having evidence that SLA and leaf-type were interchangeable variables, a two-sample t-test was accomplished to analyze the relationship between leaf-type and number of morphospecies. As predicted, mean number of morphospecies in maple leaves (5.9 ± 0.23 SE) was 23% greater compared with rhododendron leaves (4.8 ± 0.33 SE; 2-sample t-test, $t=2.74$, $df = 18$, $p = 0.013$; Figure 3). Though it wasn't statistically calculated, a majority of the plates containing maple leaves appeared to have much more growth than those containing rhododendron (Fig. 4). SLA and morphospecies number were also significantly positively correlated with each other (correlation, $r = 0.53$, $df = 18$, $p=0.016$; Figure 1).

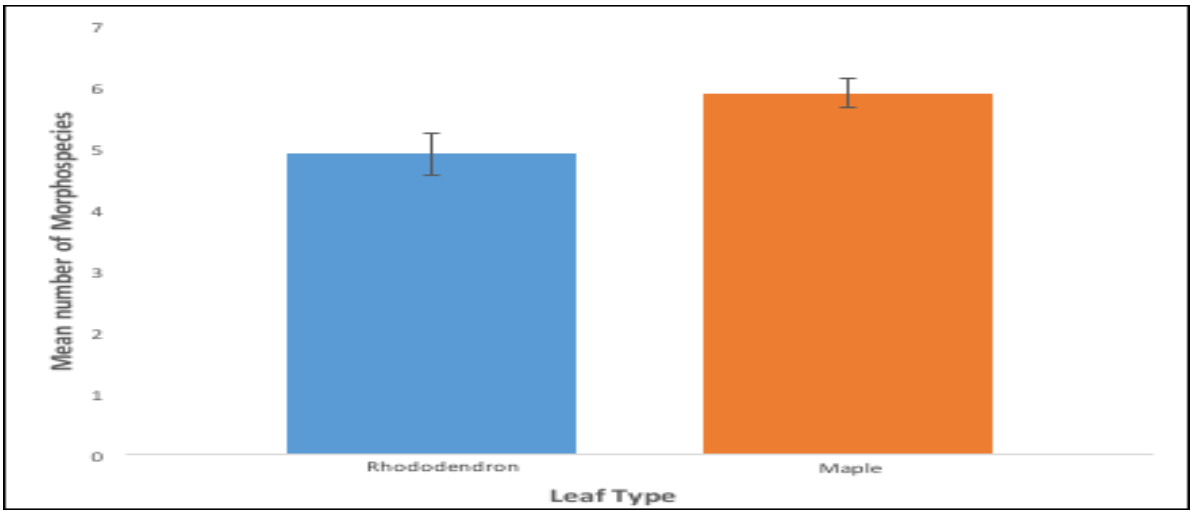


Figure 3. Effect of leaf-type on mean number of endophyte morphospecies (±1 SE). Maple leaves (n=10 leaves) had 23% greater mean number of morphospecies compared with rhododendron leaves (n=10 leaves, p=0.013).

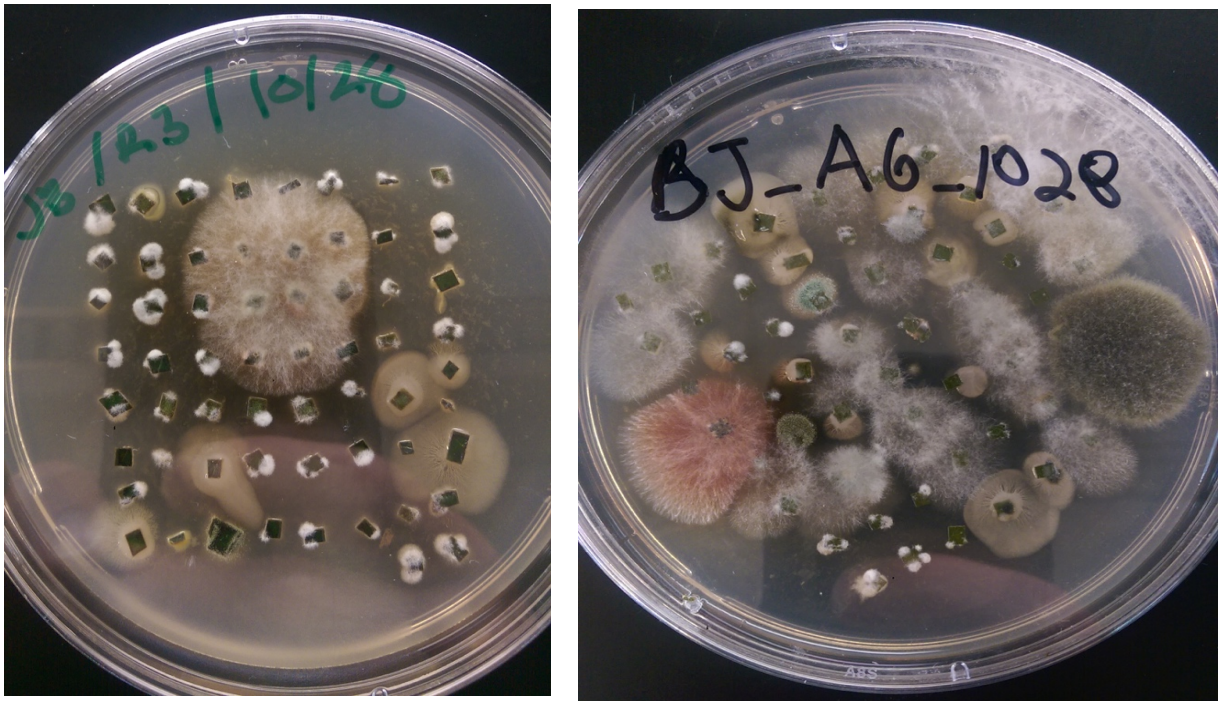


Figure 4. Morphospecies growth on 2% malt agar petri dish. R3 plate represents a sample of rhododendron leaf that showed above average growth among all 10 samples of the same leaf type. A6 on the right shows average growth among maple leaf samples and great visual diversity. Though no analysis done on proportion of growth or biomass, there does seem to be a clear difference, with maple leaf showing much more diversity and growth.

Further analysis showed that canopy cover was not associated with morphospeices (correlation, r=0.10, df=18, p=0.67). Additionally, canopy cover was the same across the leaf types (2-sample t-test, t=-0.69, df=18, p=0.50).

Discussion

This experiment sought to identify specific factors which affect endophyte diversity in plants. Maple leaves showed greater mean number of endophyte morphospecies than rhododendrons and also had significantly greater mean SLA. These results suggest that certain functional leaf traits, in this case SLA, can influence the richness of endophyte species in the leaves of plants. The study also looked at canopy cover as a causal factor in endophyte diversity. Neither leaf type showed a significant effect of canopy cover on mean morphospecies number.

Despite the fact that the initial prediction regarding a link between SLA and endophyte richness was supported, more research is necessary to identify the underlying mechanisms responsible for these results. It is possible that two mechanisms explain this relationship. The first explanation is that leaves with low SLA are investing so much in their structural defenses and as a result, they do not have to form symbiotic relationships to fill a need. This mechanism is predicated upon the assumption that the endophytes are providing a benefit. The other explanation is that leaves with high SLA are found in resource rich environments and so the plants are expending more energy into the leaves, which facilitates abundant endophyte morphospecies. While this second mechanism doesn't rule out possible beneficial effects from the endophytes, it is not dependent upon the assumption. Additionally, these two mechanisms are not mutually exclusive.

There are many other factors that must be addressed to determine what role SLA has in driving species richness. While latitude can play a factor in diversity, with tropical plants

typically exhibiting greater diversity, studies have shown that certain boreal plants can show greater than expected endophyte diversity (Higgins et al. 2007). Furthermore, another study looked at a host of leaf traits and their effects on endophyte communities (Sanchez-Azofeifa et al. 2011). This study found no significance with specific leaf weight (1/SLA) and endophyte diversity, which is in contrast to the present results. However, the researchers did find that a strong correlation exists between polyphenol levels and endophyte diversity. Their conclusion is that the age of the leaf is of utmost importance when determining effects of endophyte diversity. Young leaves have higher levels of polyphenol and anthocyanin, which are thought to act as chemical defenses against fungal infection. Therefore, as a leaf ages, it becomes more susceptible to infection and shows greater endophyte diversity (Sanchez-Azofeifa et al. 2011). We did factor in age visually and attempted to acquire young, undamaged leaves. Despite this precaution, the choice of leaf types becomes a potential problem when trying to determine the role of SLA in diversity. This is due to the fact that rhododendron leaves have a much longer lifespan than maple leaves. The study also noted that different plants have different levels of fungal defense chemicals. Understanding how important the variable of leaf age is to endophyte diversity, it is odd that the presumably much older leaf (rhododendron) had significantly less endophyte diversity.

While it is possible that SLA can function as a richness indicator for endophytes, further research needs to be done. Particularly, it would be useful to accomplish the study again while measuring the polyphenol levels of the leaves. A much better prerequisite for age estimation would also be necessary. Additionally, to ascertain whether canopy cover (as a representation of the amount of light a plant is getting) affects morphospecies richness, a sample of leafs could be chosen from high and low light areas. These leaves would provide extreme examples similar to

the SLA variable in that we would know a difference exists. Adding all these factors together, a much larger sample size should be used to see if the general trend persists, with low SLA leaves having less morphospecies and high SLA leaves having more.

The ultimate goal of future research is to extract the DNA from these morphospecies that were found and specifically identify which species are present. Many studies have begun to do this sequencing analysis and shown that the host plant can greatly affect the endophyte community, both physically by nutrient provision and genetically (Sun et al. 2014). By linking genetic analysis with factors such as leaf age, SLA and plant specificity (leaf-type), we can get a better picture of how endophyte communities are shaped and how they react with their host.

References

Aly, A. H., A. Debbab, and P. Proksch. 2011. Fungal endophytes: unique plant inhabitants with great promises. Applied Microbiology & Biotechnology 90:1829–1845.

Arnold, A. E., L. C. Mejía, D. Kyllo, E. I. Rojas, Z. Maynard, N. Robbins, and E. A. Herre. 2003. Fungal endophytes limit pathogen damage in a tropical tree. Proceedings of the National Academy of Sciences of the United States of America 100:15649–15654.

Cornelissen, J. H. C., S. Lavorel, E. Garnier, S. Diaz, N. Buchmann, D. E. Gurvich, P. B. Reich, H. Ter Steege, H. D. Morgan, M. G. A. Van Der Heijden, and others. 2003. A handbook of protocols for standardised and easy measurement of plant functional traits worldwide. Australian journal of Botany 51:335–380.

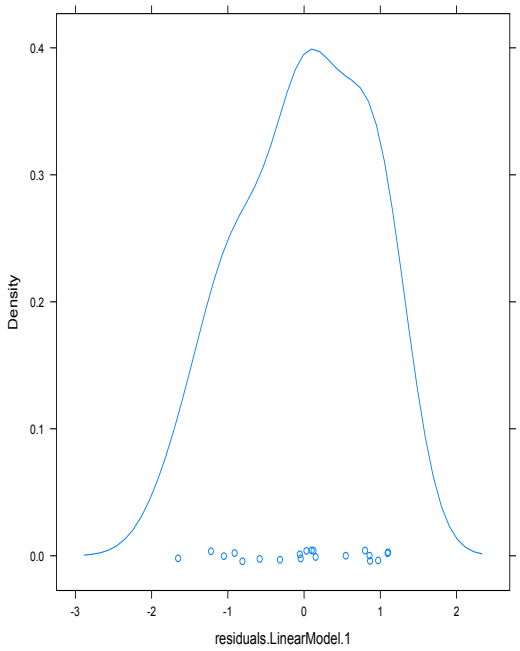
Higgins, K. L., A. E. Arnold, J. Miadlikowska, S. D. Sarvate, and F. Lutzoni. 2007. Phylogenetic relationships, host affinity, and geographic structure of boreal and arctic endophytes from three major plant lineages. Molecular phylogenetics and evolution 42:543–555.

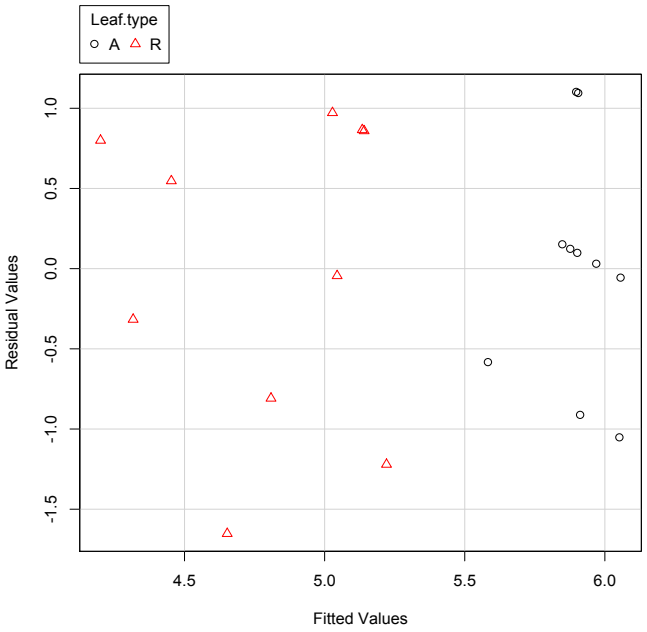
Reece, J. B., L. A. Urry, M. L. Cain, S. A. Wasserman, P. V. Minorsky, and R. B. Jackson. 2011. Campbell biology. Benjamin Cummings / Pearson, Boston.

Sanchez-Azofeifa, A., Y. Oki, G. W. Fernandes, R. A. Ball, and J. Gamon. 2011. Relationships between endophyte diversity and leaf optical properties. Trees 26:291–299.

Sun, J., F. Xia, L. Cui, J. Liang, Z. Wang, and Y. Wei. 2014. Characteristics of foliar fungal endophyte assemblages and host effective components in Salvia miltiorrhiza Bunge. Applied Microbiology & Biotechnology 98:3143–3155.

Appendix:





Analysis of Variance Table (SLA & Leaf Type)

Response: **Morphospecies**

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
SLA	1	5.7674	5.7674	7.0488	0.01729	*
Leaf.type	1	0.3572	0.3572	0.4366	0.51816	
SLA:Leaf.type	1	1.3341	1.3341	1.6305	0.21985	
Residuals	16	13.0913	0.8182			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Analysis of Variance Table (Leaf Type & SLA)

Response: Morphospecies

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Leaf.type	1	6.0500	6.0500	7.3942	0.01516	*
SLA	1	0.0746	0.0746	0.0912	0.76655	
Leaf.type:SLA	1	1.3341	1.3341	1.6305	0.21985	
Residuals	16	13.0913	0.8182			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Variable: Morphospecies

	mean	sd	se(mean)	IQR	0%	25%	50%	75%	100%	n
A	5.9	0.7378648	0.2333333	0.75	5	5.25	6	6.00	7	10
R	4.8	1.0327956	0.3265986	1.75	3	4.00	5	5.75	6	10

Variable: SLA

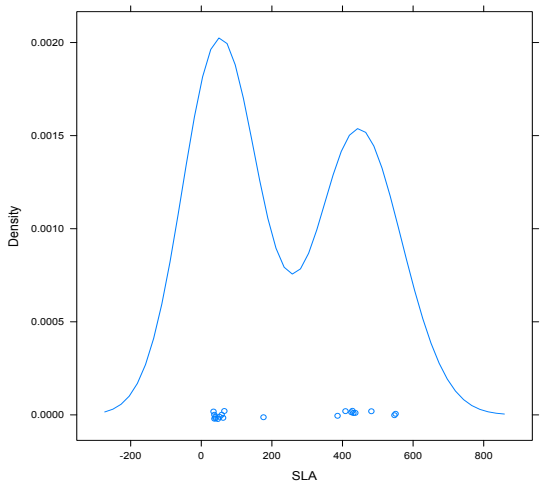
	mean	sd	se(mean)	IQR	0%	25%	50%	75%	100%	n
A	427.05	104.18390	32.945842	57.825	176.2	412.600	429.55	470.425	550.4	10
R	47.26	11.22301	3.549028	18.325	34.6	37.875	43.70	56.200	65.3	10

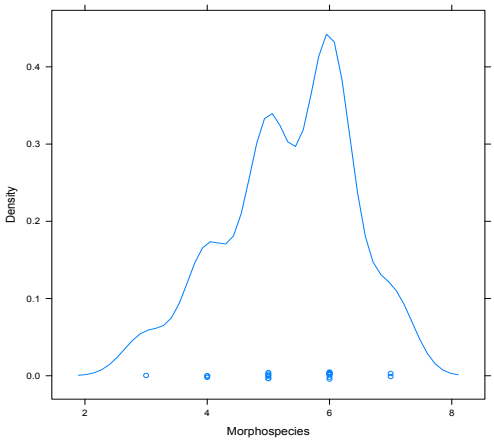
Two Sample t-test
data: SLA by Leaf.type
t = 11.461, df = 18, p-value = 1.053e-09
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
310.1729 449.4071
sample estimates:
mean in group A mean in group R
427.05 47.26

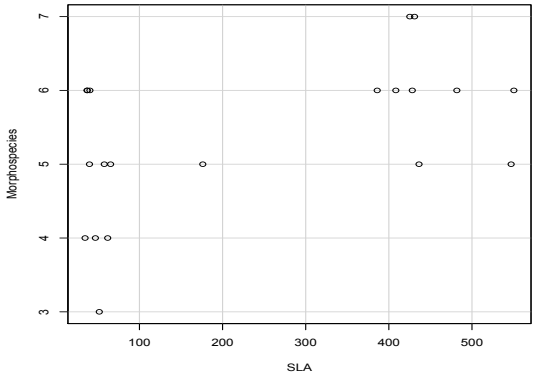
Two Sample t-test
data: Morphospecies by Leaf.type
t = 2.7405, df = 18, p-value = 0.01344
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
0.2567183 1.9432817
sample estimates:
mean in group A mean in group R
5.9 4.8

	mean	sd	se(mean)	IQR	0%	25%	50%	75%	100%	data:n
A	5.9	0.7378648	0.2333333	0.75	5	5.25	6	6.00	7	10
R	4.8	1.0327956	0.3265986	1.75	3	4.00	5	5.75	6	10

Correlation: SLA & Morphospecies
Pearson's product-moment correlation
data: Morphospecies and SLA
t = 2.65, df = 18, p-value = 0.01629
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
0.1139598 0.7876380
sample estimates:
cor
0.5297647







Pearson's product-moment correlation
data: Canopy.Cover and Morphospecies
t = 0.4349, df = 18, p-value = 0.6688
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
-0.3566411 0.5209845
sample estimates:
cor
0.1019733

Two Sample t-test
data: Canopy.Cover by Leaf.type
t = -0.69019, df = 18, p-value = 0.4989
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
-19.411103 9.811103
sample estimates:
mean in group A mean in group R
30.9 35.7