Growth of Fungal Endophytes Isolated from *Quercus macrocarpa* Roots and *Acer rubrum* Bark

Daniel Dziadula and Emma O'Malley Galvan
Department of Biology
Lake Forest College
Lake Forest, Illinois 60045

Plants harbor rich microbial communities both internally and externally. Endophytes are fungi and bacteria that colonize internal plant tissues without causing harm and may even benefit their hosts. To better understand endophyte ecology, this study compared the growth of fungal colonies isolated from Quercus macrocarpa root and Acer rubrum bark tissues under different culture conditions. Samples were collected from Lake Forest College and cultured on Potato Dextrose Agar (PDA) or Malt Extract Agar (MEA) media to test the hypotheses that 1) endophytes would exhibit greater growth (mm^2) on MEA compared to PDA and 2) endophytes coming from different tissues would have differing competitive abilities, influencing the growth they show on differing media. The results contrasted the first hypothesis as nutrient media had no effect on endophyte growth in general, or for these two specific species on these specific media. However, our second hypothesis was supported, showing significant interactions for growth between the two tissues on PDA compared to MEA. This suggests that competition between distinct species may yield significant difference in growth on various media. These findings suggest that while growth media may not affect endophyte growth uniformly, the tissue source significantly influences competitive interactions. Future research should explore the mechanisms underlying these competitive dynamics, including potential resource partitioning and allelopathic effects. This study contributes valuable insights into endophyte ecology, emphasizing the need for further investigation of their interactions across diverse plant species.

Introduction

Plants are complex in many ways, and one facet in which this can be observed is through their ecology. Many interactions take place throughout the plant body. Some may be visible, while others are not. Zooming in, plant-microorganism interactions are taking place both internally and externally throughout the plant body. These interactions can impact either organism in this relationship in various ways. Some interactions may cause harm to either of the individuals, but in other cases, both individuals may benefit, only one may, or none (Berg, 2009; Evert & Eichorn, 2012). Endophytes are fungi that occur ubiquitously within all known plants, occasionally creating mutualisms with their hosts (Sun & Guo, 2012). Recently accepted descriptions of endophytes characterize their colonization as occurring within internal plant tissues without harming the host (Compant et al., 2010; Petrini, 1991). This contrasts with epiphytes, which are characterized as organisms living on the external surfaces of the host plant (Petrini, 1991). Previous studies researching the diversity of endophytes have found that *Ascomycetes* and *Basidiomycetes*, 2 major phyla of fungi, commonly colonize deciduous and coniferous trees (Arnold et al., 2007). This means that endophytes can be found in even the most suburban neighborhoods of the United States.

Studies have shown that fungi, such as endophytes, harbor pharmaceutical potential, and being ubiquitous in all known plants, there are many reasons why the study of endophytes is warranted (Arnold et al., 2000). Endophytic fungi have also been noted to produce beneficial bioactive chemicals for their hosts and to decompose leaf litter (Sun & Guo, 2012), making them important for ecosystem services and energy recycling. Endophytes are commonly isolated utilizing sterilization techniques of the tissue sample of interest. While the epiphytes are removed, the prevailing endophytic fungi are then left to grow as colonies on nutrient-rich media, often consisting of agar. Understanding the essential growth factors that are best suited to cultivate a particular species, such as an optimal growth medium, can aid research in efficiently producing substantial endophyte colonies of interest.

Other research has shown that cultivation-dependent techniques most commonly utilize standard Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), and Sea Water Agar (Sun & Guo). Once transferred onto this media, colonies may reveal themselves to be fast- or slow-growing endophytic fungi. Growth of endophytic communities has been shown to display not only primary and secondary colonizers, but facilitation and inhibition (competition) between species as well (Saunders & Kohn, 2008). This begs the question of not only whether nutrient media affects the growth of a given species, but also whether nutrient media affects the competition of two distinct endophytic species as well.

The goal of this study was to compare the growth of two distinct endophyte colony isolates individually placed upon PDA and MEA, as well as the growth of the two species competing on each medium. It was predicted that endophyte growth would be higher on malt extract agar (MEA) than on Potato Dextrose Agar (PDA) for both root and bark isolates. It was also predicted that root and bark isolates would compete on both MEA and PDA. Previous studies have shown that Malt Extract Agar (MEA) support greater endophytes growth out of all commonly used growth media (Torta et al., 2022), so it was hypothesized that MEA would show higher average surface area growth (mm^2) than PDA. Additionally, it was hypothesized that since the bark and root tissues come from different tissues/organs of the plant body, they will have differing competitive abilities influencing the surface area (mm^2) of endophyte growth, which will influence their competition results, regardless of what media they were grown upon.

Results

2.1 Endophyte Growth for Differing Tissue Locations as a Function of Growth Medium

Statistical analyses indicated the average surface area of fungal growth at day 9 was significantly higher for bark than for root tissue, regardless of growth medium ($p = 4.09E-12$). No significant difference was found for the average surface area of growth between MEA and PDA, regardless of tissue location ($p = 0.32$). There was also no significant interaction found for the average surface area of growth between root tissue growing on the two media compared to bark tissue growing on the two media ($p = 0.32$; Figure 1).

2.2 Competition Between Endophytes from Different Tissues on Differing Media

No significant difference was found for the average surface area of growth between MEA and PDA across tissues ($p = 0.24$; Figure 1). However, fungal growth at day 9 was significantly higher from bark than from roots in both media ($p = 1.36\text{-}27$; Figure 2). Furthermore, there was a significant interaction between tissue type and media ($p = 0.01$; Figure 2).

DISCUSSION

This study aimed to compare how nutrient media may affect the growth of cultivated endophytes, alone and in competition. The data did not support the first hypothesis that cultivation on MEA would result in higher average surface area growth for either species. The results show that the nutrient media had no effect on endophyte growth in general, or for these two specific species on these specific media. This aligns with some previous work showing minimal effect of media on endophyte growth (Saunders & Kohn, 2008). However, there was a significant difference in fungal growth from different tissues, suggesting that fungi isolated from roots and bark species may exhibit different growth patterns (Sun & Guo, 2011) and perhaps grow at varying rates.

Similar to the first experiment, fungal growth was significantly

*This author wrote this paper for Biology 348: Plant Biology taught by Dr. Camilla Pizano.

higher in bark than in root endophytes during competition, again showing the distinction between these endophytes, regardless of where or what they grow on. No significant difference was found between growth on either medium, suggesting these fungal species do not exhibit nutrient media preferences. However, a significant interaction between tissues and media suggests that competition between endophytes isolated from these tree species resulted in different outcomes on PDA than on MEA. This suggests competitive abilities may vary between endophyte species or source organs. While growing strains in isolation did not distinguish media performance, biotic interactions revealed growth impacts.

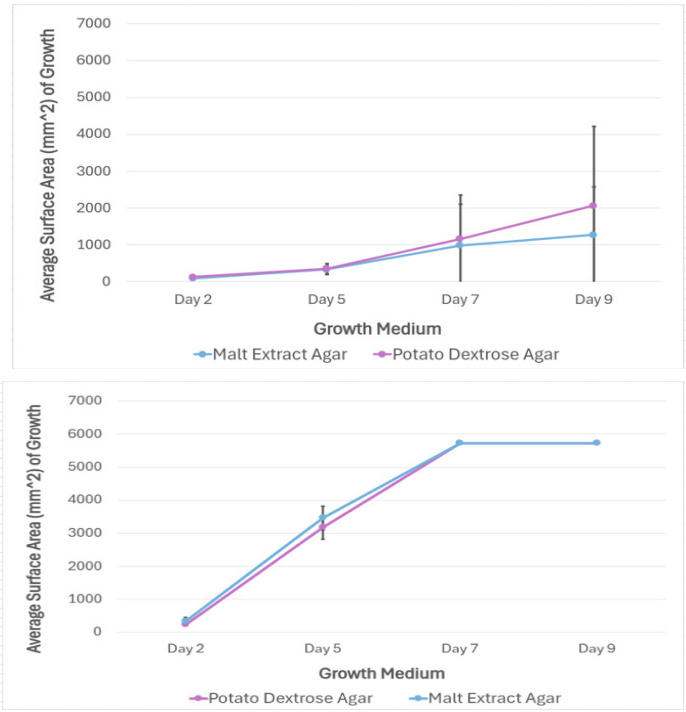

Figure 1. Average surface area (mm²) (± st. deviation) of fungal endophyte growth across 9 days as a function of growth medium for (A) *Quercus macrocarpa* root tissue and (B) *Acer rubrum* bark, with the blue line representing Malt Extract Agar (MEA) growth medium (n=10) and the pink line representing Potato Dextrose Agar (PDA) growth medium (n=10).

These findings provide insight into how interspecific competition could structure endophyte communities in plant tissues (Arnold et al., 2003). However, other mechanisms remain unclear, such as the presence/absence of stressors (Saunders & Kohn, 2008). Future work should examine competitive strategies, such as resource use or the measurement of allelopathic compound production. Identifying endophyte species and investigating interaction mechanisms may help explain their community assembly patterns.

In conclusion, this study advances understanding of endophyte population regulation by demonstrating how competition may influence growth outcomes. While not all hypotheses were confirmed, the results provide direction for additional experiments aimed at resolving the ecological forces that shape endophyte diversity and abundance within host plants. Elucidating these complex multitrophic interactions can enhance research aimed at utilizing endophytes in beneficial ways.

MATERIALS AND METHODS

4.1 Sampling Location

This experiment was originally set up on March 20th, 2024, at Lake Forest College in Lake Forest, Illinois. The sampling area was chosen by sight, upon visual confirmation of deciduous tree cover. One healthy, deciduous tree was chosen at random, which was identified as *Quercus macrocarpa* at coordinates 42□15'04"N, 87□49'40". Using gloves and sterile tools, the research team cut out a small portion of an exposed root of the tree and placed it within a sterile bag. Another healthy deciduous tree was chosen at random, which was identified as *Acer rubrum* at coordinates 42□00'08"N, 85□00'02". Using the same precautions, a small portion of the bark was collected.

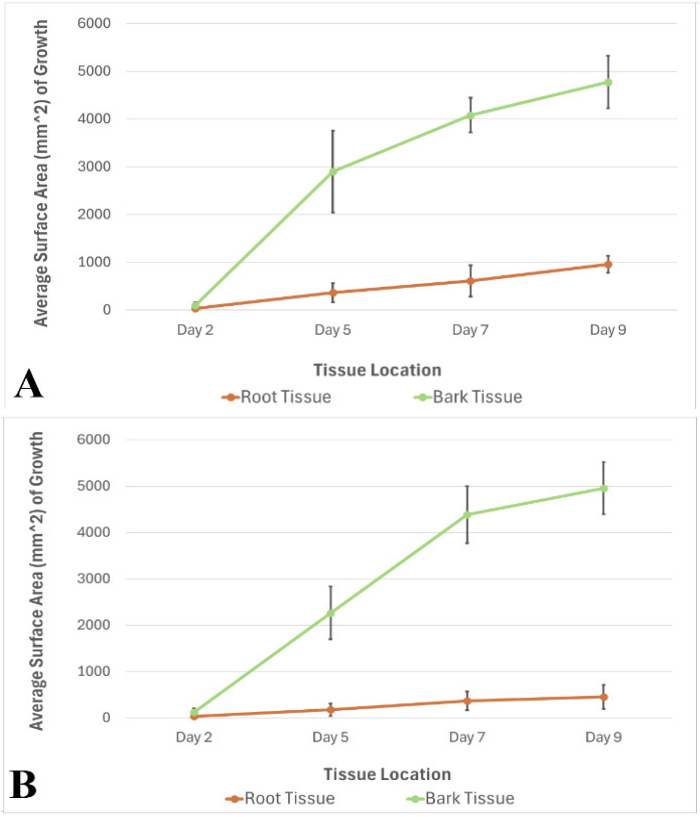

Figure 2. Average surface area (mm²) (± st. deviation) of fungal endophyte from *Quercus macrocarpa* root (n=10; brown) and *Acer rubrum* bark (green) in competition across 9 days on **(A)** Potato Dextrose Agar (PDA) and **(B)** Malt Extract Agar (MEA).

4.2 Experimental Setup

To culture endophytes, researchers must first sterilize their samples. This step is critical for isolating endophytic fungi and is commonly used in endophytic studies (Sun & Guo, 2012). The purpose of this sterilization is to eliminate epiphytic microorganisms within the sample (Petrini et al., 1992). Many studies have used these sterilization techniques to research the spatial affinities of endophytic fungi within their host plants.

Within lab 014 of Lillard Hall on Middle Campus of the college, the research team immediately prepared the samples once they were taken from the trees. To prepare the sterilization treatment, two pieces of glassware large enough to accommodate the samples were used. One glass was filled with 0.5% bleach, while the other was filled with 70% ethanol. Using sterile forceps, the samples were first submerged in 0.5% bleach for 2 minutes. Once this time had elapsed, these samples were transferred to the other glassware and submerged in 70% ethanol for 2 minutes. Following this, each sample was left to grow on Potato Dextrose Agar for 7 days.

On March 27th, 2024, these two colonies were used to create all 60 replicates for the media and media/competition experiments. Each tissue was first separated into 40 equal-sized samples. Sterilizing tweezers in bleach in between each placement, 10 replicates containing only the root tissue were placed upon PDA. Another 10 replicates were then created for the root tissue using MEA. Using a separate set of tweezers, this was repeated using the bark tissue, creating 10 replicates for PDA and 10 for MEA.

The competition experiment consisted of 10 replicates using PDA and another 10 replicates using MEA. To ensure consistent sample placement, each petri dish was marked with positioning labels 3mm from the outer edge. To ensure proper identification, the placed samples were each marked with 'R', designating root tissue, or 'B', designating bark tissue. Using the same protocol as before, 10 replicates were created upon PDA, each containing one sample of root tissue and one sample of bark tissue. This was repeated with MEA to create the 10 MEA competition dishes.

Once created, all the experimental replicates were covered with a petri dish lid, sealed, and labeled. All the replicates were left to grow within lab 014 of Lillard Hall, which is climate-controlled at room temperature.

4.3 Data Collection

After the experimental setup, data was collected by the researchers for 9 days. This took place on days 2, 5, 7, and 9, resulting in 4 data collections. To collect data, researchers spread all experimental replicates on a tabletop, grouping them by tissue and treatment. Each replicate was flipped upside down to allow researchers to determine clear boundaries for fungal growth on the agar substrate. Within the frame of each picture, a ruler marked in mm was laid out next to the experimental replicates to establish a measurement calibration for data processing. These photos were labeled by day, tissue, and treatment to ensure proper organization of the data.

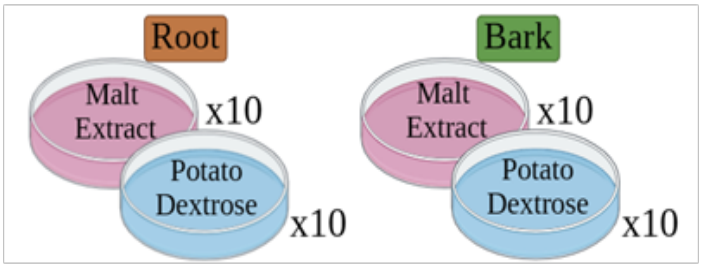

Figure 3. Setup for endophyte growth for differing tissue locations as a function of growth medium. Root and bark tissue colonies were separated into 20 equal samples each using sterilized tweezers. 10 replicates of root tissue alone were cultured on potato dextrose agar (PDA) plates. Another 10 replicates of root tissue were cultured on malt extract agar (MEA) plates. In the same manner, 10 replicates of bark tissue alone were cultured on PDA and another 10 on MEA.

4.4 Data Analysis

Data processing for this experiment was completed using the digital software ImageJ. Photos of each plate were imported into the software, and a measurement calibration was completed using the ruler present in the photos. This measurement calibration enabled the software to accurately calculate the growth's surface area. The area of growth was outlined in the photo, and the software was able to produce a measurement of the surface area in mm^2 of the fungal growth. The analysis for this experiment was completed in Microsoft Excel. Excel was used to record and calculate data, as well as to produce figures encapsulating the results. The measured growth across all 10 replicates for each treatment and tissue was used to calculate an average surface area of growth and a standard deviation for each day of data collection. These calculations were used to produce Figures A, B, C & D. The data was also used to conduct a Two-Factor ANOVA Test with replication. The first factor considered was the location of the tissue, either from the bark or root, and the second factor considered was the treatment the tissue received: MEA or PDA. This test was conducted with an alpha value of 0.05, with any p-value below 0.05 being considered a significant difference, therefore rejecting the null hypothesis that growth media had no effect on the growth of either tissue, or on the competition between them.

Acknowledgments and References

We would like to express our deepest gratitude to Dr. Pizano for her guidance and support throughout this research project. Dr. Pizano went above and beyond to help us with our experiment, provide feedback on our methods and analysis, and connect us with additional resources. Her enthusiasm for research and learning was motivating, and we could not have completed this work without her mentorship. We are also thankful for the Lake Forest College Biology Department and its staff for providing the facilities and materials necessary to conduct our laboratory experiments.

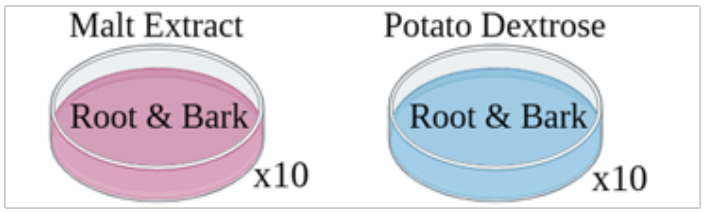

Figure 4. Setup for Endophyte Growth on Differing Growth Media as a Function of Tissue Location. 10 replicate petri dishes were prepared for each growth medium: potato dextrose agar (PDA) and malt extract agar (MEA). Within each petri dish, positioning guides 3mm from the outer edge ensured consistent placement of endophyte samples. Each sample was labeled 'R' or 'B' to designate where the endophyte came from, root or bark tissue. 10 replicates on PDA contained one sample each of root and bark endophytes. Another 10 replicates on MEA also contained a paired root and bark endophyte sample. This resulted in 20 total competition replicates to analyze potential interactive fungal growth between tissue types on the two media.

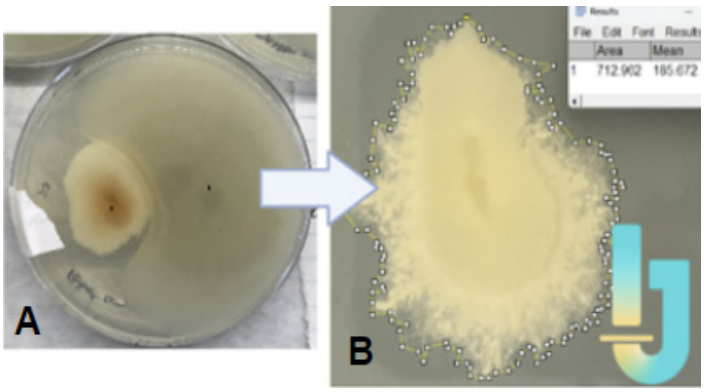

Figure 5. Measurement of fungal growth surface area using ImageJ software. A) Photograph of a petri dish containing a fungal culture plate. **B)** Screen capture from ImageJ showing digital representation of a culture plate with dotted outline indicating the measured area of interest. Photos were imported into ImageJ and calibrated with an included ruler to allow accurate area measurement determination by outlining the growth border.

References:

1. Arnold, A. E., Henk, D. A., Eells, R. L., Lutzoni, F., & Vilgalys, R. (2007). Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. *Mycologia, 99*, 185–206.

2. Arnold, A. E., Mejía, L. C., Kyllo, D., Rojas, E. I., Maynard, Z., Robbins, N., & Herre, E. A. (2003). Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences, 100*(26), 15649–15654. https://doi.org/10.1073/pnas.2533483100

3. Arnold, E., Maynard, Z., Gilbert, G., Coley, P., & Kursar, T. (2000). Are tropical fungal endophytes hyperdiverse? *Ecology Letters, 3*, 267-274.

4. Berg, G. (2009). Plant–microbe interactions promoting plant growth and health: Perspectives for Controlled use of microorganisms

in agriculture. *Applied Microbiology and Biotechnology, 84*(1), 11–18. https://doi.org/10.1007/s00253-009-2092-7

5. Compant, S., Van Der Heijden, M. G. A., & Sessitsch, A. (2010). Climate change effects on beneficial plant-microorganism interactions. *FEMS Microbiology Ecology*. https://doi.org/10.1111/j.1574-6941.2010.00900.x

6. Evert, R., & Eichorn, S. (2012). *Biology of Plants*. W.H. Freeman and Company. 8: 526-537.

7. Petrini, O. (1991). Fungal endophytes of tree leaves. In: Andrews J. H., Hirano S. S. (Eds.), *Microbial ecology of leaves* (pp. 179–197). New York: Springer.

8. Petrini, O., Sieber, T. N., Toti, L., & Viret, O. (1992). Ecology, metabolite production, and substrate utilization in endophytic fungi. *Nat Toxins, 1*, 185–196.

9. Saunders, M., & Kohn, L. M. (2008). Host-synthesized secondary compounds influence the in vitro interactions between fungal endophytes of maize. *Applied and Environmental Microbiology, 74*(1), 136–142. https://doi.org/10.1128/aem.01538-07

10. Sun, X., & Guo, L. D. (2012). Endophytic fungal diversity: Review of traditional and molecular techniques. *Mycology, 3*(1), 65–76.

11. Torta, L., Burruano, S., Giambra, S., Conigliaro, G., Piazza, G., Mirabile, G., Pirrotta, M., Calvo, R., Bellissimo, G., Calvo, S., et al. (2022). Cultivable fungal endophytes in roots, rhizomes and leaves of *Posidonia oceanica* (L.) Delile along the coast of Sicily, Italy. *Plants, 11*(9), 1139. https://doi.org/10.3390/plants11091139

12. Verma, V. C., Gond, S. K., Kumar, A., Kharwar, R. N., & Strobel, G. (2007). The endophytic mycoflora of bark, leaf, and stem tissues of *Azadirachta indica*. *Microbial Ecology, 54*(1), 119–125. https://doi.org/10.1007/s00248-006-9179-9

13. Wang, Y., & Guo, L. (2007). A comparative study of endophytic fungi in needles, bark, and xylem of *Pinus tabulaeformis*. *Canadian Journal of Botany, 85*, 911-917.