Analysis of theEnophyte Communities of Geographically Isolated Specimens of *Ginkgo Biloba*

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Endophytes of *Ginkgo biloba* from two locations separated by 65km were studied in an attempt to determine if endophytic fungi of this plant exhibit host-jumping or co-introduction. Leaves were collected from Cameron University (CU) in Lawton Oklahoma and the University of Science and Arts of Oklahoma (USAO) in Chickasha at time of abscission. Leaves were sterilized with 95% ethanol and 20% bleach solutions and plated on Rose Bengal Chloramphenicol agar. Surface washed leaves were treated with 1% Sodium Dodecyl Sulfate and then plated on Rose Bengal Chloramphenicol Agar. Permanent mounts were made using Lactophenol Aniline Blue as both the stain and mounting medium. Results suggest that host-jumping is the most likely explanation for endophyte transfer in *Ginkgo biloba*.

INTRODUCTION

Traditionally, endophytes are defined as bacteria, fungi, and algae that grow inside plant tissues without causing symptoms of disease (Elamo and others 1999; Tremouillaux-Guiller and others 2002; Shipunov and others 2008). Because of the lack of symptoms, endophytes have been a neglected portion of the plant community, but they are found in almost every plant species studied to date (Petrini 1986). Recently, the definition of an endophyte has been expanded to include mutualistic and neutral endophytes, as well as latent pathogens that can live at least part of their life cycle without causing symptoms of a disease (Duran and others 2005). Due to this extended definition and new evidence indicating that endophytism can commonly evolve from parasitism and vice-versa (Shipunov and others. 2008), the study of endophytes is receiving a great deal of attention.

A number of studies have documented that an endophyte may be harmless in some hosts and act as a pathogen in others. For example, potato blight, *Phytophthora infestans,* was harmless to several subspecies of *Solanum tuberosum,* but caused the historic potato famine that displaced and led to the deaths of millions from 1845 to 1857 (Simpson and Ogorzaly 2001). Likewise, Chestnut Blight, *Cryphonectria sp.*, only affects *Castanea dentata* (American chestnut), but that was sufficient to devastate the forests of the eastern United States during the early twentieth century (Simpson and Ogorzaly 2001). Currently concern over *Guignardia citricarpa,* Citrus black spot, is impacting the citrus industry of Argentina to the extent that the U.S. now quarantines lemons from Argentina. Despite its apparently harmless relationship within some trees, *G. citricarpa* is a dormant pathogen in others. Duran and others (2005) found that the endophytic and pathogenic conditions of *G. citricarpa* are morphologically indistinguishable. This fungus is an ideal example of the need to understand which endophytes are present within a host and how they are transferred. The transfer of endophytes is contributing to the understanding of how pathogens are transmitted. Specifically a debate exists between the co-introduction hypothesis in which an invasive, or introduced, plant colonizes its new range accompanied by endophytes from its native range; and the host-jumping hypothesis in which an invasive plant is colonized by endophytes from its new range (Shipunov and others 2008).

A study by Shipunov and others (2008) on the endophyte communities of spotted knapweed (*Centaurea stoebe* L.) of different geographic ranges was somewhat equivocal on the co-introduction hypothesis versus host-jumping. These researchers found support for host-jumping based on the presence of several novel haplotypes in the invaded range but could not rule out co-introduction because over 50% of haplotypes were present in both the native and invaded ranges. This project seeks to expand the Shipunov work by analyzing the endophyte communities of geographically isolated *Ginkgo biloba* trees.

Ginkgo biloba is an ideal subject for this work for a number of reasons. (1) The species is cultivated worldwide, which provides easy access to study samples as well as the opportunity for worldwide collaborative efforts in the future, (2) *Ginkgo* also allows for the control of time after leaf abscission as a variable; as *Ginkgo* typically goes through total leaf abscission in a period of approximately 24 hours, rather than most deciduous trees that slowly drop their leaves over a period of several weeks. This unique characteristic is immortalized is Howard Nemerov's poem "The Consent," published in *The New Yorker* in 1974 paraphrased as follows: "The ginkgo trees that stand along the walk...drop all their leaves... In one consent, and neither to rain nor to wind, but as though to time alone," (3) All species of Ginkgoales, except *G. biloba*, have been extinct since the Upper Jurassic (Chamberlain, 1935). This allows for simple identification of the study plant and sufficiently eliminates arguments over taxonomy, (4) The order dates back to at least the Permian and reached maximum diversity in the Jurassic suggesting the possibility of long established endophyte communities, and (5) *Ginkgo* is also known for its resistance to colonization of insects and parasitic fungi (except for the fungus *Phymatotrichum omniverum*) (Gifford and Foster 1989).

The purpose of this research is to test the hypothesis of Shipunov and others (2008) by comparing the endophytic communities of two specimens of *Ginkgo biloba* separated by 65 km in southwestern Oklahoma. Endophytes were isolated from leaf cuttings after natural leaf abscission. In this experiment we cultured, isolated, and identified endophytes to the level of genus to determine whether co-introduction or host-jumping is the mode of endophyte establishment in *Ginkgo biloba*.

MATERIALS AND METHODS

Study Area

Leaves were collected from a pollen producing tree located on the Cameron University (CU) main Campus in Lawton, Oklahoma on 24 November 2007, and from a pollen producing tree 65 km NNE of Lawton located at the University of Science and Arts of Oklahoma (USAO) in Chickasha, Oklahoma on 29 November 2007 and stored at 4°C in air tight plastic bags.

Whole leaves were separated into two treatments: a treatment to be washed as a control and a treatment to be surface sterilized. Isolates that were present in the washed treatment but not in the surface sterilized treatment are considered to be environmental contaminants and not endophytes. Washed leaves were washed in a 1% solution of Sodium Dodecyl Sulfate (SDS) and rinsed with sterile, distilled water until the affluent ran clear. Sterilized leaves were soaked in 70% ethanol for 4 minutes, and then rinsed with sterile, distilled water. Next, sterilized leaves were soaked in a 20% bleach solution (4% available NaOCL) and rinsed in sterile, distilled water until the affluent ran clear. Leaves were then cut into 1 cm^2 segments using flame-sterilized scissors.

Leaf pieces were then plated five to a plate on five plates for a total of 25 leaf pieces per treatment, on Rose-Bengal Chloramphenicol agar and incubated for 3-7 days at 25°C. After sufficient growth, individual colonies were sub cultured onto Sabourad's

Potato dextrose agar and incubated at 25°C until sporulation, which was generally 5-7 days. After three days of growth in pure culture, pre-sterilized cover slips were inserted into the media at an angle approaching 45° to allow the hyphae and spores to adhere to the cover slip. After sufficient growth had occurred (usually 2-4 days), cover slips with adherent hyphae were removed from the media and placed on sterilized glass slides along with 1-2 drops of lactophenol aniline blue which served as both a stain and mounting medium. Slides were then allowed to sit for 24-48 hours and then the edges of the slides were sealed with a clear finger nail polish.

Identification and taxonomy follows Barnett and Hunter, 3rd Edition, 1972. All plates were retained for examination of colony characteristics including, but not limited to; color, morphology, and growth rate. Wet mounts were made as necessary. In some cases, it became redundant to make permanent mounts for every colony. In such cases, wet mounts were made to verify the identity of the isolates as discussed below.

RESULTS

All 25 washed specimens from both localities produced colonies; CU produced 13 sterilized specimen colonies and USAO produced 11 sterilized specimen colonies. Of the sterilized treatment at CU 4 (31%) were *Gliomastix* sp. (Figure 1D), 3 (23%) were *Penicillium* sp. (Figure 1B), 2 (15%) were *Alternaria* sp. (Figure 1A), and 1 each (8%) were *Cladosporium* sp., *Geotrichium* sp. (Figure 1E), *Gliocladium* sp. (Figure 1C), and *Rhodotorula* sp. Of the sterilized treatment at USAO, 6 (55%) were *Alternaria* sp, 4 (36%) were *Aspergillus* sp. (Figure 1F), and 1 (9%) was *Gliomastix* sp. The washed treatment for both locations was completely dominated by *Penicillium* sp. suggesting that it is a dominant environmental contaminant but a relatively minor endophyte.

Statistical comparison between the two communities was done via chi-square with expected values derived from a contingency table in which columns were set as isolated genera and rows were set as the two lo-

Table 1. Diversity and Colonization Frequency of Endophytes Isolated from *Ginkgo biloba.*

CU-Sterile	**Genera**	**# of Isolates**
	Alternaria sp.	2
	Cladosporium sp.	1
	Geotrichium sp.	1
	Gliocephalum sp.	1
	Gliomastix sp.	4
	Penicillium sp.	3
	Rhodotorula sp.	1
CU-Washed		
	Gliomastix sp.	2
	Penicillium sp.	10*
USAO-Sterile		
	Alternaria sp.	6
	Aspergillus sp.	4
	Gliomastix sp.	1
USAO-Washed		
	Penicillium sp.	10*

*Five permanent mounts and five wet mounts were considered sufficient for verification. In actuality there were at least 25 instances of colonization.

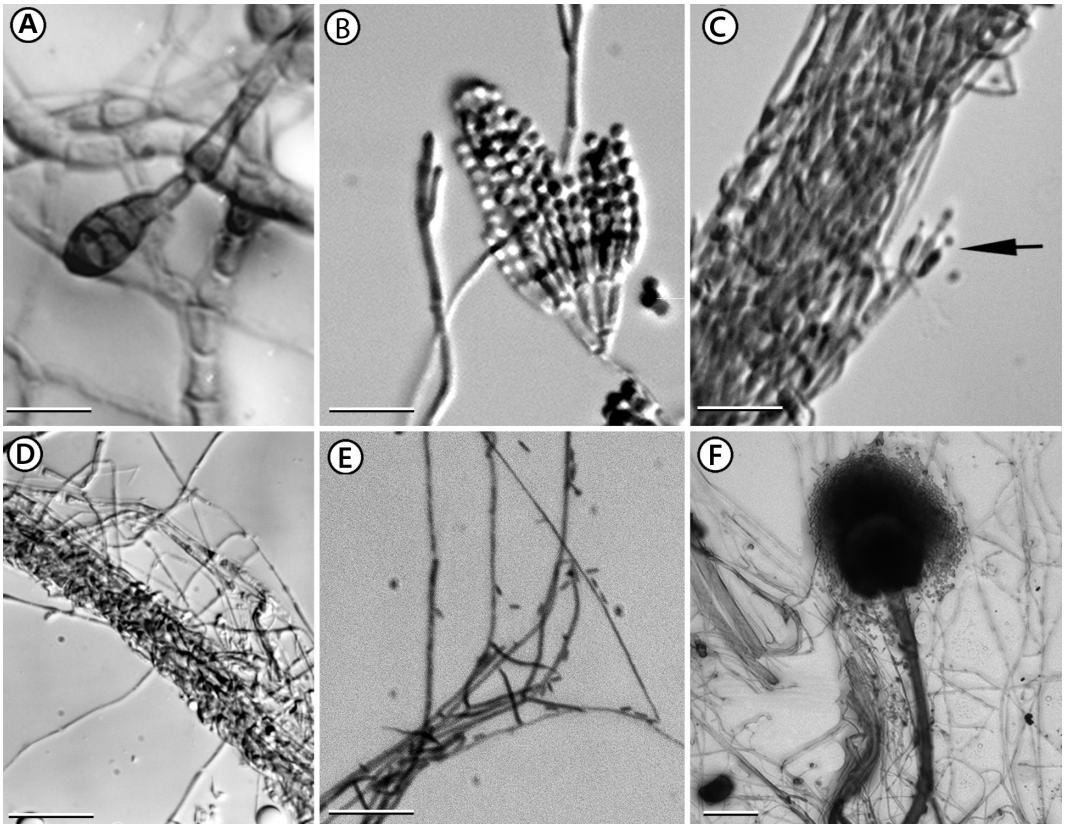

Figure 1. A-E, Scale bar = 20 μm. A) ***Alternaria sp.*** **showing conidia with transverse and longitudinal septa. B)** ***Penicillium sp.*** **showing conidiophore branched near apex bearing single celled conidia in chains. C)** ***Gliocladium sp.*** **showing penicillate conidiophore and rope-like hyphae. D)** ***Gliomastix sp.*** **characterized by rope-like aerial hyphae with conidiophores reduced to simple phialides. E)** ***Geotrichium sp.*** **with arthrospores formed by segmentation of the hyphae. F) Scale bar = 70 μm.** ***Aspergillus sp.*** **showing a conidiophore with conidial head of single celled, globose conidia.**

calities. Expected values were calculated by dividing the product of the column and the row sum by the total sum of all rows and all columns (Appendix 1). $X^2_{7, 0.05} = 82.9$, suggesting that there is a significant difference between the endophyte communities of *Ginkgo biloba* in the two locations.

Discussion

Penicillium's dominance in washed specimens from both localities suggests it is an epiphytic contaminant. However its presence on three sterilized specimens at CU suggests that this taxon may produce endophytic and epiphytic domains. Such growth architecture would give *Penicillium* a competitive advantage over other endophytes which may lack such variation (Duran and others 2005). The ability to determine whether *Penicillium* is a contaminant, an endophyte, or both, is currently beyond the scope of this work.

The significant difference between the endophyte communities of these two *Ginkgo* trees suggests host-jumping based on the presence of *Cladosporium, Geotrichium, Gliocladium,* and *Rhodotorula,* only at CU and *Aspergillus,* only at USAO. However, the four former are only found as single isolates while *Aspergillus*, with four isolates, is relatively abundant (Figure 2). This dis-

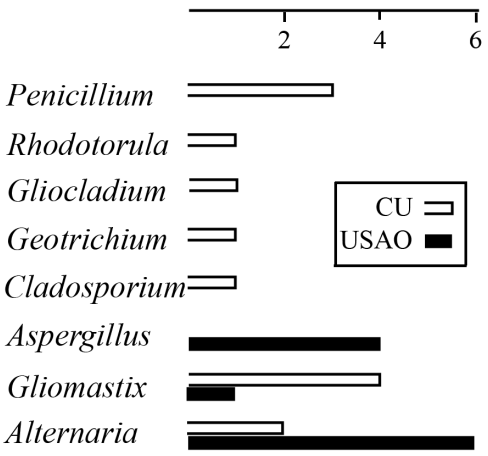

Figure 2. Analysis of endophyte communities of sterile treatments at CU and USAO. Abundance and diversity of endophytes isolated from sterile treamtnets at both CU and USAO locations. Note the genera that are exclusive to each location and the two shared genera (*Gliomastix* and *Alternaria*)

tribution is consistent with other studies of this type where a few genera are dominant and numerous others are present at lower frequencies (Shipunov and others 2008). In contrast, co-introduction can also be supported by the co-occurrence of two of the more dominant genera at both localities. For example, *Alternaria* was dominant at USAO but still found at relatively high frequencies at CU and *Gliomastix* was dominant at CU but still found at USAO. Inconclusive results such as this have also been found in all similar studies that we are aware of (i.e. Tedersoo and others 2007; White and Backhouse 2007; and Shipunov and others 2008).

Several studies, including Shipunov and others 2008, have indicated that the evolutionary lines between endophytes and pathogens are unclear and have even shown that endophytism has evolved from parasitism in *Botrytis* at least twice. Further studies of how endophytes interact with their hosts and how endophytes are transferred could prevent environmental and economic crises such as the potato blight, or more currently black citrus spot, and are currently under way (Duran and others 2005 and references therein). In additional studies it may be more effective not to use long established exotic plants such as *Ginkgo biloba* but rather to analyze relatively recent introductions of taxa such as *Bothriochloa bladhii* (Retz) S.T. Blake (Caucasian old world bluestem). Such a study could greatly contribute to our understanding of endophyte and pathogen horizontal transfer.

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Appendix 1. Calculation of Expected Values by a Standard Contingency Table

Location	**Alternaria**	**Aspergillus**	**Cladosporium**	**Geotrichium**	**Gliocladium**	**Gliomastix**	**Penicillium**	**Rhodotorula**	**Row Sum**
Cu	2	0	1	1	1	4	3	1	13
USAO	6	4	0	0	0	1	0	0	11
Column Sum	8	4	1	1	1	5	3	1	48

Expected Values

Location	Alternaria	Aspergillus	Cladosporium	Geotrichium	Gliocladium	Gliomastix	Penicillium	Rhodotorula	
Cu	2.167	1.083	0.271	0.271	0.271	1.354	0.813	0.271	
USAO	1.833	0.917	0.229	0.229	0.229	1.146	0.688	0.229	
Chi-Square	(o-e)								
CU	-0.167	-1.083	0.729	0.729	0.729	2.646	2.188	0.729	
USAO	4.167	3.083	-0.229	-0.229	-0.229	-0.146	-0.688	-0.229	
	(o-e)^2								
CU	0.028	1.174	0.532	0.532	0.532	7.000	4.785	0.532	
USAO	17.361	9.507	0.053	0.053	0.053	0.021	0.473	0.053	
	(o-e)^2/e								sum
CU	0.013	1.083	1.963	1.963	1.963	5.170	5.889	1.963	20.008
USAO	9.470	10.371	0.229	0.229	0.229	0.019	0.688	0.229	21.464
sum	9.483	11.455	2.192	2.192	2.192	5.188	6.577	2.192	
									Column sum 41.471
									total sum 82.943

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