Saprotrophic Capacity of Endophytic Fungi

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Saprotrophic Capacity of Endophytic Fungi

Emily L. Davis

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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Endophytic fungi inhabit the living tissue of a host plant for at least a portion of their life cycle. While some researchers have shown that various endophytic fungi participate in litter decomposition, we do not know whether such fungi are actually saprotrophic, meaning that they can obtain energy from litter. Therefore, I determined if endophytic fungi are saprotrophs using leaf litter as the energy source. All 49 tested isolates were found to be saprotrophic. To compare the saprotrophic capacities of fungi from different habitats, which produce different types of litter, a universal litter proxy needs to be used. I hypothesized that pure cellulose would be an adequate proxy for litter for *in vitro* studies because of its abundance in litter. This was tested in the first study. Saprotrophic capacity on pure cellulose was not highly correlated with that on leaf litter. I conclude, therefore, that cellulose may not be a good proxy for leaf litter.

Some endophytic fungi are biotrophs, presumably acquiring energy from photosynthate produced by the host plant. This suggests that the level of exposure to sunlight by the plant should influence the competitive ability of such fungi. If saprotrophic endophytic fungi do exist, they ought to be less competitive against biotrophic endophytic fungi in leaves receiving full sunlight than in shaded leaves. I, therefore, hypothesized that the frequency of saprotrophy will be influenced by the level of sun exposure of the leaf from which the fungi were isolated. This was tested in the second study. Moreover, because closely related organisms ought to be more similar to each other than more distantly related organisms, I also hypothesized that saprotrophic capacity has a strong phylogenetic component, which was also tested in the second study. Unexpectedly, isolate identity within genus accounted for far more variability in saprotrophic capacity than genus identity, and sun exposure did not have a significant effect on saprotrophy. These results suggest that saprotrophic capacity may not be highly consequential in the ecology of these organisms.

Keywords: biotrophy, cellulose, decomposition, endophytic fungi, *in vitro* methods, litter, *Quercus gambelii*, saprotrophic capacity, saprotrophy
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CHAPTER ONE: Evaluating the use of cellulose for *in vitro* saprotrophy studies

**Abstract**

Endophytic fungi inhabit the living tissue of a host plant for at least a portion of their life cycle. While previous research has shown that some endophytic fungi participate in litter decomposition, no one has yet determined whether these fungi are able to obtain energy from decomposing litter or, in short, whether they are saprotrophic. To compare the saprotrophic capacities of fungi from different habitats, which produce different types of litter, a universal litter proxy needs to be used. I hypothesized that pure cellulose would be an adequate proxy for litter for *in vitro* studies because of its abundance in litter. I compared the saprotrophic capacities of endophytic fungal isolates on pure cellulose with that on leaf litter. The linear correlations of both the actual saprotrophic capacities as well as the rank-orders of the saprotrophic capacities were low. Therefore, I concluded that pure cellulose is a poor proxy for litter in assessing saprotrophy. Still, I found that some endophytic fungi are saprotrophic. The ecological implications of this are discussed.

**Keywords:** cellulose, decomposition, endophytic fungi, *in vitro* methods, litter, *Quercus gambelii*, saprotrophy

**Introduction**

Saprotrophic fungi acquire nutrition from decaying organic material (Boddy 2016). All saprotrophic fungi are decomposers (Lindahl & Tunlid, 2014) and, therefore, play an important role in important ecosystem processes such as humus formation (Coûteaux *et al.*, 2005), soil carbon sequestration (Cotrufo *et al.*, 2013), and nutrient cycling (Boddy & Watkinson, 1995).
While endophytic fungi are generally considered to be biotrophic, evidence suggests that some endophytic fungi are saprotrophic and, therefore, would be the first fungi to decompose litter after host tissue death (Müller et al., 2001; Promputtha et al., 2007; Sun et al., 2011; Szink et al., 2016; Jia et al., 2020).

Litter mass loss is a common measurement for decomposition (Bärlocher, 2020). However, it is not an appropriate measure for saprotrophy because decomposers do not necessarily obtain nutrition from decomposing litter. Saprotrophy is best measured as growth of the organism when given litter as its energy source. This can be straightforward in many cases. However, valid comparison of the saprotrophic capacities of fungi from contrasting habitats may be quite difficult. The available energy sources in a dry pine forest, for example, may be quite different from those in a wet peat bog. When comparing the saprotrophic capacities of fungi from contrasting habitats such as these, what would the appropriate substrate be? Pine needles may be an appropriate substrate for assessing saprotrophy of pine forest fungi, but pine needles may not be appropriate for assessing the saprotrophic capacity of peat bog fungi because of the potential for specialization of fungi with respect to the peculiar chemistries of particular sources of litter (Ayres et al., 2009; Chomel et al., 2016).

There is, therefore, a need for a well-defined proxy for litter than can be used in comparative studies of saprotrophy. Pure cellulose, for example, may be a practical alternative because it is abundant in litter. Some researchers have utilized pure cellulose when studying decomposition (Uchida et al., 2005; Güsewell & Gessner, 2009; Tiegs et al., 2013), although others have doubted its utility (Fritz et al., 2011) because it does not account for the complex chemistry of most litter types (Chomel et al., 2016). Therefore, my goal was to evaluate whether
pure cellulose could serve as proxy for litter when comparing the saprotrophic capacities of endophytic fungal isolates.

**Methods**

*Study sites and leaf sampling*

Szink et al. (2016) provided indirect evidence that many of the endophytic fungi from leaves of *Quercus gambelii* Nutt. (Gambel’s oak), a common tree species in Utah, are saprotrophic. For this study, I tested endophytic fungi isolated from unblemished leaves of *Quercus gambelii*. *Quercus gambelii* leaves were first sampled in Slate Canyon (40.13’30.59”N, 111.37’21.9”W), elevation 1553 m, in Provo, Utah, and at Devil’s Kitchen (39.48’12.27”N, 111.41’17.96”W), elevation 2553 m, located near Payson, Utah. These sampling locations represent much of the elevational range of *Q. gambelii*. At Slate Canyon, I collected 20 leaves from each of 6 *Quercus gambelii* trees. At Devil’s Kitchen, I collected 15 leaves from each of 7 trees of *Quercus gambelii*. All leaves, from both Slate Canyon and Devil’s Kitchen, were placed into plastic sandwich bags, immediately stored in a cooler with ice, and transferred to a 5°C refrigerator in the laboratory within two hours.

*Fungal isolation*

I sterilized external leaf surfaces by dipping leaves into 70% ethanol, immediately placing them into 3% sodium hypochlorite for 2 minutes (Arnold et al., 2000), removing the hypochlorite by dipping leaves sequentially into three beakers of sterile water, and dipping leaves again into 70% ethanol. They were then laid to air dry in a sterile, paper-lined petri plate.
I then removed a subsample from each leaf using a sterilized paper hole punch. The subsample was placed on 2% malt extract agar in petri plates and sealed with parafilm. The plates were stored in an incubator at 17°C, which approximated an average growing season temperature of the two sampling sites. If multiple isolates grew from a single leaf subsample, each was subcultured. After growth of each fungal isolate was established and the mycelium reached a diameter of about 18 mm, plates were stored at 5°C until use.

**Experimental treatments**

Forty-nine isolates were chosen for testing based on differences in color, hyphal growth pattern, and hyphal density. Replicates of each isolate were grown on three types of media: control medium and two treatment media containing either ground leaf litter or cellulose as the main carbon sources. The control medium contained 200 mL distilled water, 0.4g glucose, 1.784 g agar, 0.092 g peptone, 0.2 ml of 300 g CaCl₂ L⁻¹, 2.0 ml of 30g KH₂PO₄ L⁻¹, 0.2 ml of 5 g MgSO₄ L⁻¹, 3.7 g FeSO₄ L⁻¹, 1.4 g MnSO₄ L⁻¹, and 3.7 g ZnSO₄ L⁻¹. In addition to the components of the control medium, the leaf medium contained 1.436 g *Quercus gambelii* leaves milled with a Cyclone Mill Twister (Retsch, Haan, Germany) to pass a 2 mm opening. The milled leaf litter in the leaf medium provided a total carbon concentration equivalent to half-strength potato dextrose agar. The leaves were collected during summer 2020 from the forest floor at the Slate Canyon site. In addition to the components of the control medium, the cellulose medium contained 1.436 g microcrystalline cellulose (BulkSupplements.com, Henderson, NV, USA).

For each of the 49 isolates, there were four replicate cultures grown on each of the three media. The plates were wrapped in parafilm and placed in an incubator at 17°C, which
approximated an average growing season temperature of the two sampling sites. After approximately 50 days, the fungal mycelium was removed from each plate along with the agar medium, and placed individually into separate 8 cm diameter, stainless steel tea balls. The agar was melted in boiling water for 10 minutes, and the fungal mycelium was removed from the tea ball, dried on the lab bench overnight, and weighed. I defined saprotrophic capacity to be the difference between growth rate on leaf medium and control medium, or the difference between growth rate on cellulose medium and control medium.

Statistics

For each isolate, to calculate the error associated with the difference between growth rate on treatment vs. control media (saprotrophic capacity), the differences were calculated for all 16 possible combinations (four replicates of each treatment). Four of the 16 differences were then randomly sampled with replacement 1000 times to bootstrap a frequency distribution of the difference, and the mean was determined in R (R Core Team, 2018). The R script is given in the supplementary materials.

Pearson’s correlation coefficient, $r$, (SigmaPlot, Systat Software, San Jose, CA) and Spearman’s correlation coefficient, $\rho$, (Minitab, 2010) were calculated for the relationship between saprotrophic capacity on leaf medium and saprotrophic capacity on cellulose medium. Pearson’s $r$ characterizes the linear correlation between the saprotrophic capacities on both treatment media, while $\rho$ characterizes the linear correlation between the rank-orders of the saprotrophic capacities on both media.

Results
The linear relationship between the saprotrophic capacities on leaf and cellulose media, and the linear relationship between the rank-orders of the saprotrophic capacities on leaf and cellulose media had significant slopes. Pearson’s $r$ was 0.494, and the $R^2$ value was 0.244 (Figure 1). Spearman’s $\rho$ was 0.631.

**Discussion**

The Pearson correlation coefficient of 0.494 indicates that the saprotrophic capacity on cellulose medium had a positive linear association with saprotrophic capacity on leaf medium. However, the $R^2$ of 0.244 indicates that only about 24% of the variability in saprotrophic capacity on leaf medium was accounted for by variability in saprotrophic capacity on cellulose medium. Therefore, for any particular fungal isolate, saprotrophic capacity on cellulose did not appear to be a good proxy for saprotrophic capacity on leaf litter.

The Spearman’s $\rho$ of 0.631 indicates that there was a positive correlation between rank-order of saprotrophic capacity on the two media. In other words, the ranking in terms of saprotrophic capacity on cellulose was similar to the ranking in terms of saprotrophic capacity on leaves but, again, for any particular isolate the ranking on cellulose medium was not a good indicator of the ranking on leaf medium.

The poor correlation between growth on cellulose and growth on leaf litter is likely due to the chemical complexity of litter coupled with variation among isolates in their ability to handle non-cellulosic litter components such as tannins and lignin (Bending & Read, 1997; Osono, 2007, 2020). High tannin concentrations, such as occur in oak leaves (Feeny, 1970), cause enzymes to become less active (Benoit 1968), and lignin is generally more difficult to
decompose than cellulose (Berg, 1991; Berg & Ekbohm, 1991; Fioretto et al., 2005). There may be instances when using pure cellulose would be appropriate as a litter proxy, such as when cellulolytic ability per se is of interest (Deacon, 1979). However, it would appear that cellulose is not a good proxy for chemically complex litter.

Nonetheless, this is the first time that endophytic fungi have been shown to be saprotrophs. This may not be true for every endophytic fungus, which, instead, may be obligate biotrophs. At host tissue senescence, however, endophytic fungi that are saprotrophic would have priority over non-endophytic fungi when decomposing and acquiring nutrition from litter. This priority would consequently affect subsequent fungal decomposer communities (Cline & Zak, 2015; Lin et al., 2015; Song et al., 2017) and decomposition rates (Cline et al., 2018; Jia et al., 2020; Wolfe & Ballhorn, 2020; Fanin et al., 2021).
Figure 1. Linear correlation of the saprotrophic capacities of 49 isolates on cellulose medium versus saprotrophic capacities on leaf medium.
Supplementary Material

Bootstrapping R script

```r
install.packages("boot", dep=TRUE)
library(boot)

treatment.vec = c(# growth rates of all 4 replicates of one isolate grown on leaf medium)
glucose.vec = c(# growth rates of all 4 replicates of isolate grown on glucose medium)
treatment.vec
.glucose.vec

combinations = expand.grid(treatment.vec, glucose.vec)
combinations

treatment.comb = combinations[c(1:1)]
glucose.comb = combinations[c(2:2)]
treatment.comb
glucose.comb

difs = treatment.comb - glucose.comb
difs.vec = difs[,1]

BootstrapMean = function(X = difs.vec) {
x.boot = sample(X, size = 4, replace = T)
mean(x.boot)
}

N = 1000
boot.replicate = replicate(1000, BootstrapMean())
stat = rep(NA, N)
for (i in 1:N) {
stat[i] = BootstrapMean()
}

par(mfrow = c(1, 2))
hist(stat)
abline(v = mean(difs.vec), lwd = 2, col = "red")
hist(boot.replicate)
abline(v = mean(difs.vec), lwd = 2, col = "red")

BootstrapMean()
mean(difs.vec)
mean = mean(stat)
mean
stddev = sd(stat)
SE = (sd(stat)/sqrt(4))
SE
```
error = qnorm(0.975)*stddev/sqrt(4)
left = mean-error
right = mean+error
list (mean, stddev, left, right)
REFERENCES


Cline LC, Zak DR. 2015. Initial colonization, community assembly and ecosystem function:


CHAPTER TWO: Variation in saprotrophic capacity of endophytic fungi

Abstract

Endophytic fungi inhabit the living tissue of a host plant for at least a portion of their life cycle. My previous study (chapter 1) was the first to document saprotrophy in endophytic fungi. In the current study, my goal was to document the sources of variation among isolates of endophytic fungi in saprotrophic capacity. Some endophytic fungi are biotrophic, presumably acquiring energy from photosynthate produced by the host plant. This suggests that the level of exposure to sunlight by the plant should influence the success of biotrophic fungi and, therefore, their competitive ability against saprotrophic endophytic fungi. I, therefore, hypothesized that the frequency of saprotrophy among endophytic fungi will be influenced by the level of sun exposure of the leaf from which the fungi were isolated. Moreover, because closely related organisms ought to be more similar to each other than more distantly related organisms, I also hypothesized that saprotrophic capacity would have a strong phylogenetic component. Unexpectedly, isolate within genus accounted for far more variability in saprotrophic capacity than genus, and sun exposure did not have a significant effect on saprotrophy. This suggests that saprotrophic capacity may not be highly consequential in the ecology of these organisms.

Keywords: biotrophy, endophytic fungi, litter, Quercus gambelii, saprotrophic capacity, saprotrophy

Introduction

All terrestrial plant species investigated thus far form symbioses with endophytic fungi (Arnold et al., 2000; Rodriguez et al., 2009). By definition, these fungi spend at least a portion of
their life cycle within the living tissues of a host plant (Rodriguez et al., 2009; Porras-Alfaro & Bayman, 2011; Koide, 2019). The fungal symbionts range from pathogens and parasites (e.g. Fisher & Petrini, 1992; Redman Regina et al., 2001; Romero et al., 2001; Rodriguez et al., 2009; Swinfield et al., 2012) to mutualists (e.g. Malinowski et al., 2000; Redman Regina et al., 2001; Redman et al., 2002; Arnold et al., 2003; Jaber & Vidal, 2010; Li et al., 2012; Zarea et al., 2012; Sangamesh et al., 2018). Often, a single endophytic fungal species is capable of multiple types of symbiosis, depending on host plant identity and environmental conditions (Carroll, 1988; Redman Regina et al., 2001; Faeth & Fagan, 2002; Arnold, 2007; Rodriguez et al., 2009; Swinfield et al., 2012). The structure of endophytic fungal communities can be influenced by host plant identity (Kivlin et al., 2019; Frani et al., 2020), host plant age (Arnold et al., 2003; Helander et al., 2006; Osono, 2008), season (Osono, 2008), and climate (Hoffman & Arnold, 2008; Giauque & Hawkes, 2013).

When an endophytic fungus grows or reproduces exclusively within the tissues of a host plant, the fungus must acquire necessary resources from the host plant and, therefore, is biotrophic (Faeth & Fagan, 2002; Kemen & Jones, 2012; Delaye et al., 2013; Koide, 2019). Clavicipitaceous endophytic fungi (Clay, 1990; Rodriguez et al., 2009), as well as Class 2 and Class 4 endophytic fungi are examples of biotrophs as they grow extensively within host plant tissues (Rodriguez et al., 2009). Obligate biotrophs, such as some mycorrhizal fungi, must complete their life cycle while the host plant is alive (Lewis 1973). To maintain biotrophic endophytic fungi, host plants must produce adequate amounts of photosynthate (Konvalinková & Jansa, 2016) for both themselves and their biotrophic fungi. This suggests that, in similar environments, biotrophic fungi will be more vigorous in host plants that receive considerable solar radiation than in those receiving little solar radiation. If there is competition between
biotrophic and saprotrophic endophytic fungi for space, we might expect the frequency of saprotrophy among fungal isolates to be lower in leaves exposed to full sun than in those in the shade.

Indeed, endophytic fungi are not necessarily all efficient biotrophs. In fact, Class 3 endophytic fungi have limited ability to grow in host plants (Rodriguez et al., 2009) suggesting that they are not efficient biotrophs, if biotrophic at all. Instead, they may simply be waiting until the tissue dies to obtain energy saprotrophically (Koide, 2019). As a whole, then, endophytic fungi may have a wide range of trophic status. This mirrors the trophic diversity observed in other plant-associated fungi. For example, ectomycorrhizal fungal species exist along a biotrophy-saprotrophy continuum (Koide et al., 2008).

Circumstantial evidence suggests that some endophytic fungi are saprotrophic (Promputtha et al., 2007; Szink et al., 2016; Jia et al., 2020). Many endophytic fungal species have been found in decomposing litter (Szink et al., 2016), and some have been shown to be capable of litter decomposition (Müller et al., 2001; Sun et al., 2011). This is not surprising. Litter is an immense source of reduced carbon for saprotrophs (Schlesinger, 1977) and it seems reasonable that natural selection would favor the evolution of endophytism among certain lineages of saprotrophic fungi, allowing them to colonize plant tissues prior to their death and, therefore, establishing priority over saprotrophs that cannot colonize live tissue (Koide, 2019). Endophytic fungi have already been shown to affect decomposition rates, providing circumstantial evidence that this priority exists (Cline et al., 2018; Jia et al., 2020; Wolfe & Ballhorn, 2020; Fanin et al., 2021).

Therefore, in this study I tested the following hypotheses: (1) Some endophytic fungal isolates are saprotrophic; (2) Saprotrophic capacity varies among endophytic fungal isolates; (3)
Saprotrophic capacity varies more among genera than within a genus; and (4) Saprotrophic frequency is lower in leaves exposed to full sun than those in shaded leaves.

**Methods**

*Study sites and leaf sampling*

For this study, I used endophytic fungi sampled from unblemished leaves of *Quercus gambelii* Nutt. (Gambel’s oak), a common tree species in Utah occurring along a wide range of elevation. Sampling sites were located in Slate Canyon (40.13’30.59”N, 111.37’21.9”W), elevation 1553 m, in Provo, Utah, and at Devil’s Kitchen (39.48’12.27”N, 111.41’17.96”W), elevation 2553 m, near Payson, Utah, representing much of the elevational range of the species. At Slate Canyon, I collected 20 leaves from each of 6 *Quercus gambelii* trees. Ten of the leaves from each tree were sampled from the outside of the crown and therefore received maximum sun exposure. The other 10 leaves were sampled from the interior of the crown and, therefore, received minimal sun exposure. At Devil’s Kitchen, I sampled 15 leaves from each of 7 trees of *Quercus gambelii*, all from the outside of the crown. All leaves, from both Slate Canyon and Devil’s Kitchen, were placed into plastic sandwich bags, immediately stored in a cooler with ice, and transferred to a 5°C refrigerator in the laboratory within two hours.

*Fungal isolation*

I sterilized external leaf surfaces by dipping leaves into 70% ethanol, immediately placing them into 3% sodium hypochlorite for 2 minutes (Arnold et al., 2000), removing the hypochlorite by dipping leaves sequentially into three beakers of sterile water, and dipping leaves again into 70% ethanol. I then placed the sterilized leaves on a sterile, paper-lined petri plate to air dry.
I removed a subsample from each leaf using a sterilized paper hole punch. The subsamples were placed on 2% malt extract agar in petri plates and sealed with parafilm. The plates were stored in an incubator at 17°C, which approximated an average growing season temperature of the two sampling sites. If multiple isolates grew from a single leaf subsample, each was subcultured separately. After fungal growth on each subculture plate was established and the mycelium reached a diameter of about 18 mm, plates were stored at 5°C until use.

Identification of fungal isolates

Fungal isolates were grouped on the basis of color, hyphal growth pattern and hyphal density. Direct PCR (Luo 2002) was performed on at least one representative isolate from each group using live hyphae as a template, mixed with APEX 2 Hotstart Master Mix (Genesee Scientific, San Diego, CA, USA), PCR water, and ITS1F and ITS4 primers (Gardes and Bruns 1993) (IDT, Coralville, IA, USA). The thermal cycling program included activation at 95 °C for 15 min followed by 30 cycles of denaturation (95°C, 30 s), annealing (55°C, 30 s), and extension (72°C, 48 s), then ended with a final extension (72°C, 7 min). Amplicons were cleaned using exonuclease I and shrimp alkaline phosphatase (New England Biolabs, Ipswich, MA, USA), and sent to the BYU DNA Sequencing Center for Sanger sequencing (Applied Biosystems 3730xl DNA Analyzer). Sequences were viewed with CodonCode Aligner (CodonCode Corporation, Centerville, MA, USA, 2019), and I used the NCBI database to identify fungal species with a 96% identity using BLASTn (Madden 2002). Species identification of each isolate used is listed in Supplementary Material Table S1.

Determining saprotrophy
Forty-nine isolates (Table S1) were chosen for testing based on differences in appearance. To determine saprotrophic capacity, replicates of each isolate were grown on two types of agar. The control medium contained 200mL distilled water, 0.4g glucose, 1.784g agar, 0.092g peptone, 0.2ml of 300g CaCl\textsubscript{2} L\textsuperscript{-1}, 2.0 ml of 30g KH\textsubscript{2}PO\textsubscript{4} L\textsuperscript{-1}, 0.2ml of 5g MgSO\textsubscript{4} L\textsuperscript{-1}, 3.7g FeSO\textsubscript{4} L\textsuperscript{-1}, 1.4g MnSO\textsubscript{4} L\textsuperscript{-1}, and 3.7gZnSO\textsubscript{4} L\textsuperscript{-1}. The leaf medium contained the same materials plus 1.436g Quercus gambelii leaves milled to pass a 2 mm opening with a Cyclone Mill Twister (Retsch, Haan, Germany). The milled leaves provided a total carbon concentration equivalent to half-strength potato dextrose agar. The leaves were collected during summer 2020 from the forest floor at the Slate Canyon site.

For each of the 49 isolates, there were four replicate cultures grown on leaf medium and four replicate cultures grown on control medium. The plates were wrapped in parafilm and placed in an incubator at 17°C, which approximated an average growing season temperature of the two sampling sites. After approximately 50 days, the fungal mycelium was removed from each plate along with the agar medium, and placed individually into separate 8 cm diameter, stainless steel tea balls. The agar was melted in boiling water for 10 minutes, and the fungal mycelium was removed from the tea ball, dried on the lab bench overnight, and weighed. I considered saprotrophic capacity to be the difference between growth rate on leaf medium and growth rate on control medium.

Statistical methods

For each isolate, to calculate the error associated with the difference between growth rate on leaf medium vs. control media (saprotrophic capacity), the differences were calculated for all 16 possible combinations (four replicates of each treatment). Four of the 16 differences were
then randomly sampled with replacement 1000 times to bootstrap a frequency distribution of the
difference, and the mean and 95% confidence interval was determined in R (R Core Team,
2018). The R script is given in the supplementary materials. I defined an isolate as a saprotroph
when its saprotrophic capacity was significantly larger than zero based on the 95% confidence
interval.

To test the hypothesis that the saprotrophic capacity among endophytic fungal isolates
varies, I analyzed the variation among all 49 isolates without respect to taxonomy or phylogeny
using analysis of variance (n=4) performed at this website: https://acetabulum.dk/anova.html,
using the means and standard deviations calculated in R (above).

To test the hypothesis that saprotrophic capacity varies more among genera than within a
genus, a second analysis of variance was performed to determine the effect of genus, using
isolates within a genus as replicates, in Minitab (Minitab, 2010). A third analysis of variance
performed to determine the effect of isolate within a single genus (Ophiognomonia) using the
website: https://acetabulum.dk/anova.html, using the means and standard deviations calculated in
R (above).

It was not necessary to test the hypothesis that the frequency of saprotrophy among
fungal isolates is lower in those isolated from leaves exposed to full sun than in those isolated
from shaded leaves because all isolates were saprotrophic (see Results below). Instead, I
performed an analysis of variance of saprotrophic capacity using only data from the isolates from
Slate Canyon. The single factor in the analysis was sun exposure to determine whether
saprotrophic capacity is, on average, lower in isolates sampled from leaves exposed to full sun
than those sampled from shaded leaves. To control for phylogeny, another analysis of variance of
saprotrophic capacity was performed using data solely from *Ophiognomonia* isolates from Slate Canyon.

**Results**

*Identification of fungal isolates*


*Saprotrophic status*

No isolate had a 95% confidence interval that encompassed zero (Figure 2). Therefore, I considered all the isolates to be saprotrophic.

*Variation in saprotrophic capacity*

Isolate was a significant source of variability in saprotrophic capacity among the 49 isolates (Table 1). According to the sums of squares in the analysis of variance, 95% of the total
variability occurred among isolates and only 5% of the variability occurred within an isolate (Table 1). There was a 183-fold variation in saprotrophic capacity among the 49 isolates (Figure 2).

Genus was not a significant source of variability in saprotrophic capacity among the 4 genera comprising multiple isolates (Figure 3, Table 2). According to the sums of squares in the analysis of variance, only 6% of the total variability was due to variation among genera and 94% of the variability was due to isolate within a genus (Table 2). The mean saprotrophic capacity for each genus is listed in Table 3.

Within the genus Ophiognomonia, isolate was a significant source of variability in saprotrophic capacity (Figure 4, Table 4). In fact, the isolates with the least and greatest saprotrophic capacities were in this genus. Thus, the genus Ophiognomonia contained essentially as much variation as occurred among all isolates.

Influence of sun exposure

All isolates were considered saprotrophic. Therefore, leaf sun exposure did not affect the frequency of saprotrophy among isolates. Moreover, for all the isolates from Slate Canyon, leaf sun exposure was not significant with respect to isolate saprotrophic capacity (Table 5). Even when the analysis was restricted to isolates from a single genus, Ophiognomonia, leaf sun exposure was also not significant with respect to fungal saprotrophic capacity (Table 6), indicating that sun exposure did not affect the saprotrophic capacity of isolated endophytic fungi, even when much of the phylogenetic variation was eliminated.
Discussion

The results show that all the endophytic fungal isolates tested are saprotrophs. Saprotrophic fungi that are endophytic would already be established in the plant tissue when the tissue dies, and therefore would have priority over saprotrophs that cannot colonize the tissues of a living plant. They are the first organisms that can decompose plant litter. This priority effect is likely to influence subsequent decomposer fungal community succession (Cline & Zak, 2015; Lin et al., 2015; Song et al., 2017) and, therefore, may affect nutrient cycling and ecosystem productivity (Flanagan & Van Cleve, 1983; Attiwill & Adams, 1993; Saikkonen et al., 2015; Jia et al., 2020; Wolfe & Ballhorn, 2020).

While isolate identity was a significant source of variation, genus was not significant. In fact, the isolates with the least and greatest saprotrophic capacities were from the genus *Ophiognomonia*, meaning that within this genus, there was as much variation as existed among all 49 isolates. Because I did not have multiple isolates of each species tested, I could not analyze variability at the species level. But it is quite interesting that there was more variability within a genus than among genera. Of the total variability observed, 94% was attributed to the variability within a genus, while only 6% was attributed to the variability among genera. One usually expects individuals within a genus to be more similar to each other than to individuals in another genus, or, in other words, conservation of ecologically important traits (Powell et al., 2009; Giauque et al., 2019). The isolates tested were from four different classes yet had quite similar saprotrophic capacities. This suggests that this trait may be of little ecological importance. It may also be that 50 days simply wasn’t long enough to characterize the ecologically relevant saprotrophic capacities of these isolates. Had the experiments been extended to 100 days,
saprotrophic capacities may have been different. Still, we were specifically examining endophytic fungi as initial litter decomposers, so 50 days may have been sufficient.

Solar radiation may affect the community composition of foliar endophytic fungi (Koide et al., 2017) as well as decomposer fungi in litter (Duguay & Klironomos, 2000; Pancotto et al., 2003). However, I have shown that solar radiation affected neither the frequency of endophytes that were saprotrophic nor their saprotrophic capacities. In retrospect, this may not be all that surprising. It is possible that no Class 3 endophytic fungi are efficient biotrophs and, therefore, variation in solar radiation may have no impact on their competitive ability. Consequently, the level of solar radiation may not affect their interactions with purely saprotrophic endophytic fungi. In fact, the results indicate that some endophytic fungi are not necessarily obligate biotrophs, but may actually be facultative biotroph-saprotrophs or even obligate saprotrophs that have merely evolved to colonize living host tissues to insure priority access to litter upon tissue senescence (Koide, 2019).

These results undoubtedly do not apply to all endophytic fungi. They may not even apply to all endophytic fungi in Quercus gambelii leaves because I may not have isolated all endophytic fungi from Quercus gambelii. Season may influence the species composition of endophytic fungal communities (Osono & Takeda, 2002), and so when I sampled at the onset of autumn, the leaves may not have contained all the fungal species that occur within Quercus gambelii leaves. It is also possible that some endophytic fungi living within the Quercus gambelii leaves at the time of sampling were not culturable with the media used, or were not culturable with any media simply because they are obligate biotrophs. Therefore, I cannot determine how common saprotrophy is among endophytic fungi of Quercus gambelii. Still, all
the isolates I did successfully isolate were saprotrophic, which is consistent with the hypothesis that at least some Class 3 endophytic fungi are saprotrophic.
Tables and Figures

Figure 2. Mean saprotrophic capacities for all 49 isolates. The error bars are 95% confidence intervals.

Table 1. ANOVA table for variation in saprotrophic capacities among the 49 isolates (see Figure 1).

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate</td>
<td>48</td>
<td>78.7684</td>
<td>1.64101</td>
<td>56.9127</td>
<td>3.33e-73</td>
</tr>
<tr>
<td>Error</td>
<td>147</td>
<td>4.2386</td>
<td>0.02883</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>195</td>
<td>83.007</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3. Mean saprotrophic capacities for all 49 isolates, grouped by genus. Red = *Apiognomonia*, Blue = *Cladosporium*, Green = *Coniochaeta*, Yellow = *Ophiognomonia*, and White = genera containing a single isolate. The latter were not included in the analysis of variance in Table 2.

Table 2. ANOVA table for variation in saprotrophic capacities among the 4 genera comprising multiple isolates (see Figure 3).

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among genera</td>
<td>3</td>
<td>1.076</td>
<td>0.3586</td>
<td>0.93</td>
<td>0.437</td>
</tr>
<tr>
<td>Within genus</td>
<td>38</td>
<td>14.694</td>
<td>0.3867</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>15.77</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Table of means of saprotrophic capacities among the 4 genera comprising multiple isolates.

<table>
<thead>
<tr>
<th>Genus</th>
<th>N</th>
<th>Mean</th>
<th>SE Mean</th>
<th>Tukey Grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Apiognomonia</em></td>
<td>4</td>
<td>0.923</td>
<td>0.311</td>
<td>A</td>
</tr>
<tr>
<td><em>Cladosporium</em></td>
<td>4</td>
<td>0.771</td>
<td>0.311</td>
<td>A</td>
</tr>
<tr>
<td><em>Coniochaeta</em></td>
<td>2</td>
<td>1.104</td>
<td>0.44</td>
<td>A</td>
</tr>
<tr>
<td><em>Ophiognomonia</em></td>
<td>32</td>
<td>0.55</td>
<td>0.11</td>
<td>A</td>
</tr>
</tbody>
</table>
Figure 4. Mean saprotrophic capacities for the 32 isolates of Ophiognomonia. The error bars are the 95% confidence intervals.
Table 4. ANOVA table for variation in saprotrophic capacities among the 32 isolates of Ophiognomonia (see Figure 4).

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate</td>
<td>31</td>
<td>44.486</td>
<td>1.435</td>
<td>66.1192</td>
<td>5.36e-52</td>
</tr>
<tr>
<td>Error</td>
<td>96</td>
<td>2.0836</td>
<td>0.0217</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>127</td>
<td>46.5695</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5. ANOVA table for variation in saprotrophic capacities due to sun exposure among the 33 isolates from Slate Canyon.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sun exposure</td>
<td>1</td>
<td>0.1659</td>
<td>0.1659</td>
<td>0.42</td>
<td>0.522</td>
</tr>
<tr>
<td>Error</td>
<td>31</td>
<td>12.2587</td>
<td>0.3954</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>12.4246</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6. ANOVA table for variation in saprotrophic capacities due to sun exposure among the 22 isolates of Ophiognomonia from Slate Canyon.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>1</td>
<td>0.2502</td>
<td>0.2502</td>
<td>1.12</td>
<td>0.303</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>4.4787</td>
<td>0.2239</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>4.7289</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
SUPPLEMENTARY MATERIALS

Bootstrapping R script

install.packages("boot",dep=TRUE)
library(boot)

treatment.vec = c(#growth rates of all 4 replicates of one isolate grown on leaf medium)
glucose.vec = c(#growth rates of all 4 replicates of isolate grown on glucose medium)
treatment.vec
glucose.vec

combinations = expand.grid(treatment.vec,glucose.vec)
combinations

treatment.comb = combinations[c(1:1)]
glucose.comb = combinations[c(2:2)]
treatment.comb
glucose.comb

difs = treatment.comb-glucose.comb
difs.vec = difs[,1]

BootstrapMean = function(X=difs.vec){
x.boot=sample(X, size=4, replace=T)
mean(x.boot) }

N = 1000
boot.replicate = replicate(1000, BootstrapMean() )
stat = rep(NA, N)
for (i in 1:N){
stat[i] = BootstrapMean()}

par(mfrow=c(1,2))
hist(stat)
abline(v=mean(difs.vec), lwd=2, col="red")
hist(boot.replicate)
abline(v=mean(difs.vec), lwd=2, col="red")

BootstrapMean()
mean(difs.vec)
mean = mean(stat)
mean
stddev = sd(stat)
SE = (sd(stat)/sqrt(4))
SE
error = qnorm(0.975) * stddev / sqrt(4)
left = mean - error
right = mean + error
list (mean, stddev, left, right)
Table S 1. Isolate name and species identity.

<table>
<thead>
<tr>
<th>Isolate name</th>
<th>Species name</th>
</tr>
</thead>
<tbody>
<tr>
<td>QG2.10</td>
<td><em>Ophiognomonia quercus-gambelii</em></td>
</tr>
<tr>
<td>QG2.9</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QG3.12.A.1</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QG3.3.A</td>
<td><em>Cladosporium</em> sp.</td>
</tr>
<tr>
<td>QG3.6.A.1</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QG4.1.R1</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QG4.9.A.2</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QG5.12.A.1</td>
<td><em>Tricharina cretea</em></td>
</tr>
<tr>
<td>QG5.4.A</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QG5.8.A.2</td>
<td><em>Saccothecium rubi</em></td>
</tr>
<tr>
<td>QG5.9.A</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QG6.6.A.1</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QG6.7.A</td>
<td><em>Cladosporium herbarum</em></td>
</tr>
<tr>
<td>QG7.10</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QG7.10.A</td>
<td>Unknown Helotiales</td>
</tr>
<tr>
<td>QG7.10.A.3</td>
<td><em>Cladosporium</em> sp.</td>
</tr>
<tr>
<td>QGshd1.10.1</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QGshd1.7.A.4.1</td>
<td><em>Ophiognomonia quercus-gambelii</em></td>
</tr>
<tr>
<td>Code</td>
<td>Species</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>QGshd1.7.A.4.2</td>
<td><em>Ophiognomonia</em> sp.</td>
</tr>
<tr>
<td>QGshd1.9.A.2.1.1</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QGshd1.9.A.2.1.2</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QGshd1.9.A.R2</td>
<td><em>Cladosporium sinuosum</em></td>
</tr>
<tr>
<td>QGshd2.3.A.2</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QGshd3.4.A.1</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QGshd3.6.2</td>
<td><em>Apiognomonia errabunda</em></td>
</tr>
<tr>
<td>QGshd3.9.A.2</td>
<td><em>Apiognomonia errabunda</em></td>
</tr>
<tr>
<td>QGshd4.4.A.2</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QGshd5.3.A.1</td>
<td><em>Ophiognomonia quercus-gambelii</em></td>
</tr>
<tr>
<td>QGshd5.9.A.1</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QGsun1.6.A.2</td>
<td><em>Ophiognomonia quercus-gambelii</em></td>
</tr>
<tr>
<td>QGsun1.8.A.1.1</td>
<td><em>Ophiognomonia</em> sp.</td>
</tr>
<tr>
<td>QGsun2.2.A</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QGsun2.2.A.1</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QGsun2.9.A.3</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QGsun3.10.2</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QGsun3.10.A</td>
<td><em>Fimetariella rabenhorstii</em></td>
</tr>
<tr>
<td>QGsun4.2.A</td>
<td>Unknown Venturiaceae</td>
</tr>
<tr>
<td>Code</td>
<td>Species</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>QGsun4.5.A.1</td>
<td><em>Apiognomonia errabunda</em></td>
</tr>
<tr>
<td>QGsun4.9.A.2.1</td>
<td><em>Pyronema omphalodes</em></td>
</tr>
<tr>
<td>QGsun4.9.A.3</td>
<td><em>Ophiognomonia sp.</em></td>
</tr>
<tr>
<td>QGsun5.2</td>
<td><em>Parafenestella vindobonensis</em></td>
</tr>
<tr>
<td>QGsun5.3.A</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QGsun5.3.A.2</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QGsun5.5.A.1</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QGsun5.5.A.2.2</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
<tr>
<td>QGsun5.7.A.4</td>
<td><em>Coniochaeta sp.</em></td>
</tr>
<tr>
<td>QGsun6.6.A</td>
<td><em>Coniochaeta polymorpha</em></td>
</tr>
<tr>
<td>QGsun6.8</td>
<td><em>Apiognomonia errabunda</em></td>
</tr>
<tr>
<td>QGsun6.9</td>
<td><em>Ophiognomonia setacea</em></td>
</tr>
</tbody>
</table>
REFERENCES


Duguay KJ, Klironomos JN. 2000. Direct and indirect effects of enhanced UV-B radiation on


**Hoffman MT, Arnold AE. 2008.** Geographic locality and host identity shape fungal endophyte


