An Endophyte alters biological characteristics of the grass, *Festuca thurberi*

Part I: Does endophyte symbiosis alter decomposition along altitudinal gradients?

Part II: How does endophyte symbiosis affect host survival and growth?

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Abstract

Plant-fungal symbioses are found in ecosystems worldwide, but relatively little is known about how these two organisms affect each other. This study sought to shed light on this topic. The first experiment tested to see if the presence of an endophyte (genus *Epichloë*) effected the decomposition rates of its host grass (*Festuca thurberi*) and on fungal composition in litter, and whether or not the effect is different along elevation gradients. The second experiment tested for survival, growth and biomass differences between plants naturally associated with an endophyte, plants naturally endophyte-free, and plants which have had their endophyte removed using the fungicide Benomyl.

Results thus far have shown that the presence of an endophyte has had a negative effect on plant biomass and survival over the course of the four years since the experiment was began, which agrees with findings of past studies. Endophyte status does not appear to play a significant role in determining free-living fungal associate diversity among plants. However, fungal composition significantly differed between live and dead leaf tissue.

Upon the conclusion of this study, we will have gained a greater understanding of ecology of grasses and their symbionts, how endophyte symbioses affect other fungal taxa (decomposers), and how all of these species interactions affect overall ecosystem functioning.

Part I: Does endophyte symbiosis alter decomposition along altitudinal gradients?

Introduction

Global climate change is poised to alter biological systems in many ways. By the year 2100 it is estimated that the world will see a 2-4.5°C rise in mean annual temperatures, resulting in consequences such as shifts in patterns of precipitation (Solomon et al. 2007), plant respiration (Ryan 1991), food production (Parry et al.

2004), hydrology (Arnell & Reynard 1996; Christensen et al. 2004), and biodiversity (Sala et al. 2000; Xu et al. 2009).

One method of testing how biological systems may respond to shifts in climate is the use of elevation gradients to mimic the effects of warming (Fukami & Wardle 2005; McCain & Colwell 2011; Sundvquist et al. 2013). Using gradients has proven to be a useful method of study as mountains occur on all seven continents; thus elevation can be replicated both regionally and worldwide (Callaway et al. 2002; Harsch et al. 2009; McCain 2009). Past studies have shown many biological activities changing in response to elevation, including plant biomass and aboveground net primary production (NPP) (Whittaker et al 1974; Raich et al. 1997), nutrient cycling (Lovett & Kinsman 1990; Jacot et al. 2000a; Groffman et al. 2009), and community range (Whittaker 1956; Sanders et al. 2007; Bahram et al. 2012).

Plant-symbiotic endophytic fungi are found almost everywhere on the planet, with a diversity rivaling that of insects (Caroll 1988; Arnold et al. 2000), and are estimated to inhabit 20-30% of all grass species (Leuchtmann 1992). These fungi provide various services to their hosts including protection from herbivory (Clay 1996), pathogen damage (Arnold et al. 2003), and abiotic stress (Malinowski & Belesky 2000; Song et al. 2012) in exchange for organic materials. Fungal symbioses may also be important in mitigating abiotic stresses caused by climate change (Marks & Clay 1996; Kivlin et al. 2013), as well as altering ecosystem processes and characteristics such as forest succession (Rudgers et al. 2007) and species diversity (Rudgers & Clay 2008).

The decomposition rates of plant litter along elevation gradients have also garnered recent interest. Rates have been found to be higher at lower elevations due to warmer temperatures (Wang et al. 2009; Salinas et al. 2011). However, this process can be species or location-specific (Shaw and Harte 2001). For example, in Colorado, USA, Englemann spruce needle decomposition decreased linearly with elevated temperature (ie lower elevation) due to a 63% loss of soil moisture. The same study also found that total ecosystem carbon decreased ~50% with a rise in temperature due in part to a decline in dead wood (Kueppers & Harte 2005). Regardless, the majority of evidence suggests that nutrient release from litter usually occurs at a faster rate at lower elevations (Vitousek et al. 1994), which enhances the supply of nutrients from the soil and can drive further biological activity. However this pattern is not always the case (e.g., Murphy et al. 1998), likely due to an overriding influence of factors other than temperature, such as precipitation (Vitousek et al. 1994) and plant functional traits (Vitousek et al. 1988; Salinas 2011).

Despite the growing knowledge regarding altitudinal patterns of decomposition and fungal endophytes, little is known about the role of this interaction at different elevations. This study asks the question: Does the presence of an endophyte (genus *Epichloë*) affect the rate at which its host grass (*Festuca thurberi)* decomposes across altitudinal gradients? Although some previous work found that endophyte-infected (E+) litter decomposed slower than endophyte-free (E-) litter (Omacini et al. 2004; Lemons et al. 2005), there has yet to be any study into how different elevations might affect this interaction. This is an important

factor to consider regarding decomposition because it could allow for further understanding of how it might be affected by global temperature increases, as well as how climate may alter the effects of symbionts on carbon cycling in ecosystems (Iqbal et al. 2013). A change in this process in the face of climate change has the potential to alter ecosystem functioning, but the role of fungal symbionts in this context remains poorly understood. We hypothesize that the decomposition rate of E+ litter will be slower than that of E- litter across elevations. To better understand the microbial diversity associated with this community of *F. thurberi*, we also cultured fungi from leaves and tillers of both E+ and E- plants. We expected to see significant differences in this diversity between both tissue type and endophyte status.

Methods

Study species. *F. thurberi* is a densely tufted, perennial cool-season grass with blades 6-20 cm long, 1.5-3 cm wide when flat (Shaw 2008). It can be found in meadows, dry, rocky slopes and hills, and open forests in montane and subalpine regions of Southern Wyoming, Utah, Colorado, and New Mexico (Darbyshire and Pavlick 2007). The *Epichloë* species under study was recently discovered by Dr. Jennifer Rudgers and has not yet been named. It lives only in live grass hosts (Kuldau et al. 1997); thus, any effects of this endophyte on litter decomposition will be due to its legacy effect on the composition of other microbial associates present in the litter, rather than to a direct effect of a live endophyte. It has also been found that the abundance of *Epichloë* endophytes decreases with elevation (Figure 1).

Study sites. A total of three altitudinal transects were run up three separate mountains, one along Avery Peak, the second along Cinnamon Mountain, and the third along Treasury Mountain (Figure 2 and Table 1). Study plots were placed every ~200 meters starting from the base of each mountain for a total of six plots per transect.

Litter bag construction. Each study plot was given six litter decomposition bags: three experimental bags containing 5g of E+ *F. thurberi* litter, and the three control bags containing 5g of E- *F. thurberi* litter following the methods of Shaw and Harte (2001). The bags were constructed from nylon window screening (4 cm X 10 cm) with sewn edges, and then closed with plastic quilting staples. Litter for the experimental bags contained a mix of litter from 10 E+ *F. thurberi* individuals, while the controls contained the litter of 10 E- *F. thurberi* to maintain similar genetic variation between treatments. The litter was collected from naturally occurring plants at a site near the Rocky Mountain Biological Laboratory (RMBL) (38.96255014, -106.9852277; elevation 2992 m). The litter originated from naturally occurring plants near the RMBL that was scored for endophyte presence using aniline blue lactic acid stain following Bacon and White (1994).

Timing of collection. One experimental bag and one control bag will be collected from each deployment site at each of three preselected collection times: September 2014, July 2015, and August 2015. Following collection, bags will be air-dried then weighed to the nearest 0.0001 g. Decomposition rate will be calculated, and a general linear model will test how decomposition rate is affected by elevation

(continuous factor), endophyte-presence (categorical factor), and the interaction between elevation and endophyte-presence.

A potential problem involved in our approach is the use of litter from naturally occurring E+ and E- *F. thurberi*. In order to be able to directly determine causality, it would be best to culture the endophytes in a lab setting and inoculate them onto endophyte-free plants as well as performing experimental removal of the endophyte. By using naturally occurring material, there is no way to definitively know that fungal presence is the driving factor behind our decomposition rates. However, due to the exploratory nature of the project, the results retrieved will still be suggestive of the endophyte's effect on decomposition and will help us to determine whether time- and labor-intensive manipulations may be worth pursuing.

Fungal cultures. In addition to field manipulation, we cultured fungi from litter and live leaves in a lab using sterile technique under a Labconco Purifier Logic+ Class II, Type A2 biological safety cabinet. We grew cultures on potato dextrose agar plates containing penicillin and streptomycin to suppress bacterial growth. We subcultured via hyphal tipping onto separate plates as fungi emerged from the tissue. After two weeks, a PERMANOVA analysis was run using EcoSim software to understand how well fungal morphotypes differed between plate groups.

Results/Discussion, Part I

(Results of decomposition experiment forthcoming)

Fungal cultures. Tissue type (tiller vs. litter) played a significant role (p=0.0004) in determining fungal morphotype composition differences between plates (Figure 2), while endophyte status (E+ vs. E-) did not.

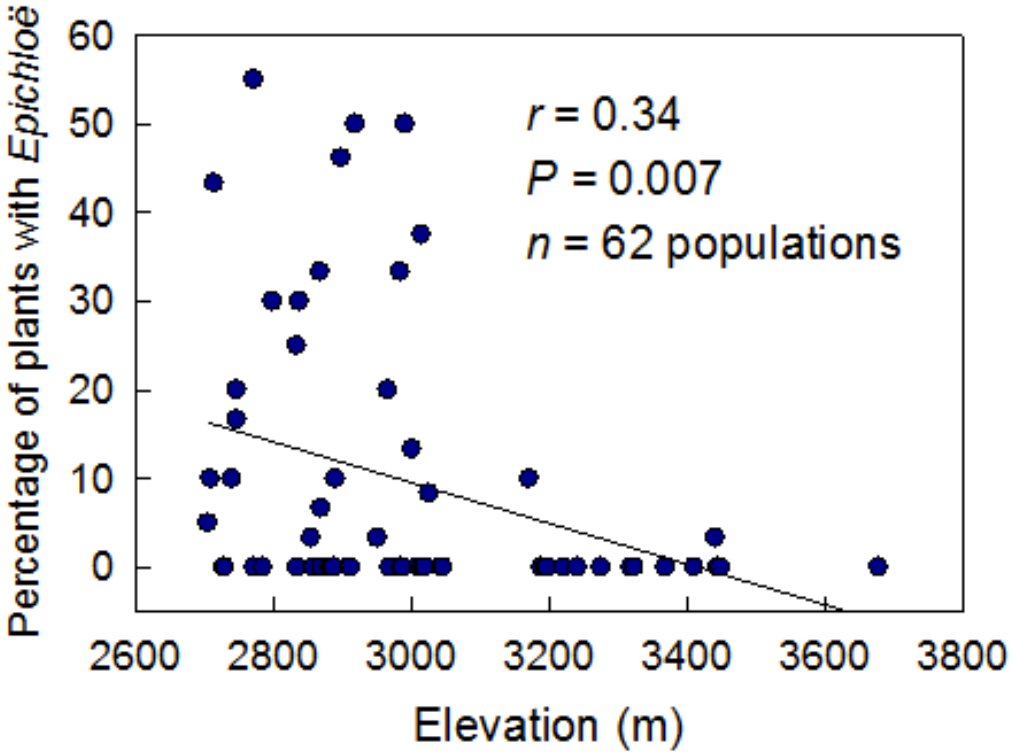

Figure 1: Altitudinal pattern in endophyte frequency in *Festuca thurberi* from data collected across 62 populations sampled between 2011 and 2013. For each population, a minimum of 12 individual plants was scored for endophyte presence using aniline blue stain on thin sections of the inner leaf sheath.

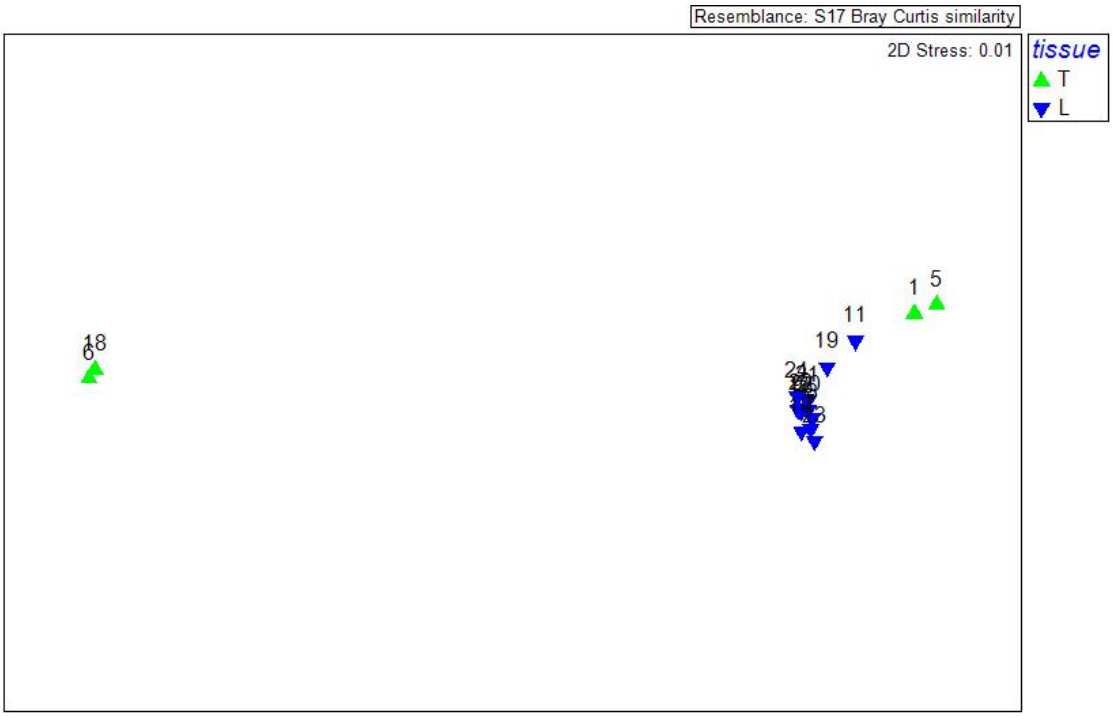

Figure 2: Relative similarities in fungal community composition among cultures and between plates

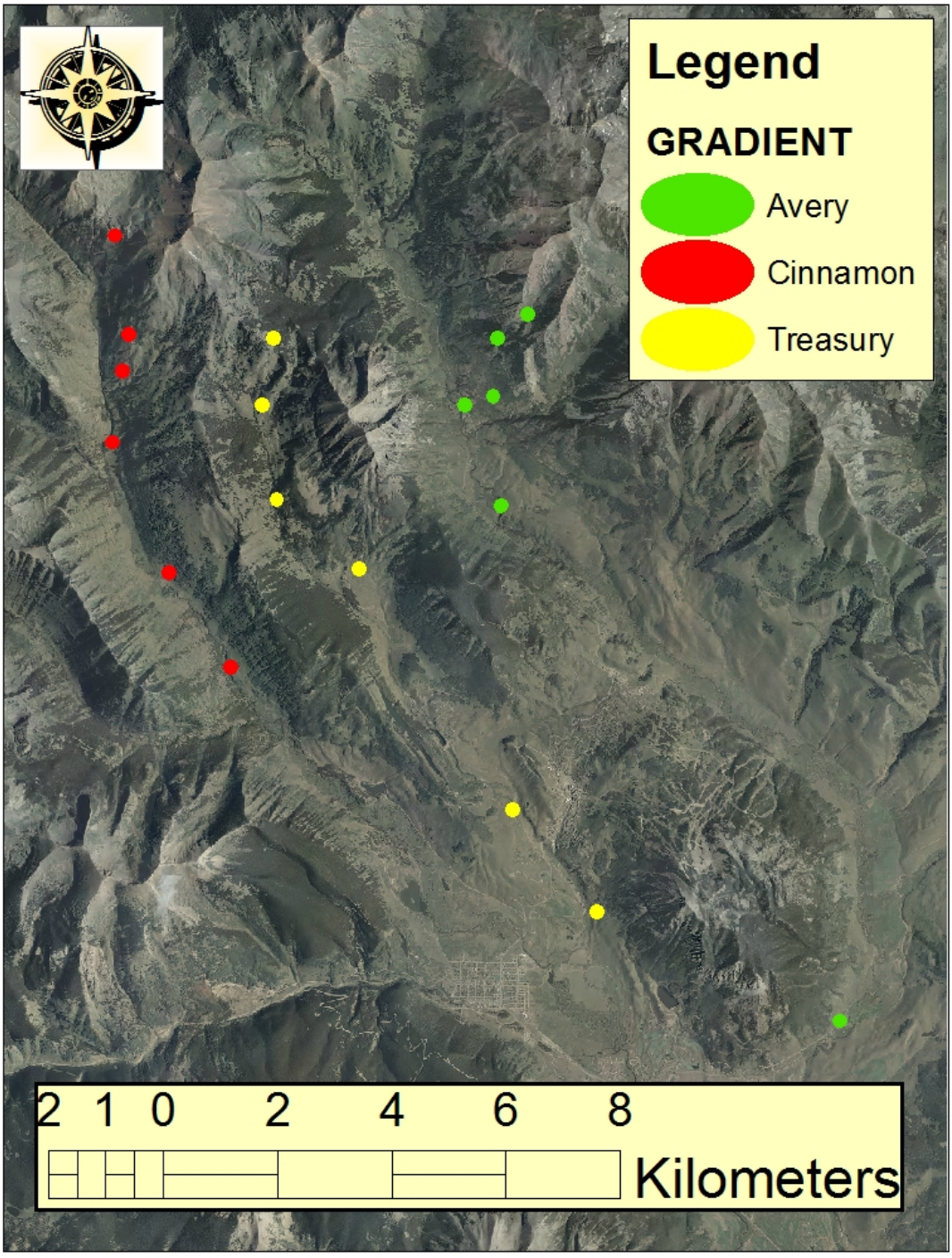

Figure 3: Litter bag deployment sites

Gradient	Latitude	Longitude	Elevation (m)
Avery Peak	38.8652	-106.9124	2732
	38.9451	-106.9828	2812
	38.9608	-106.9905	2899
	38.9623	-106.9849	2996
	38.9715	-106.9842	3192
	38.9752	-106.9783	3344
Cinnamon Mountain	38.8974	-106.9793	2798
	38.8816	-106.9618	2746
	38.9347	-107.0112	2956
	38.9454	-107.0281	3044
	38.9601	-107.0314	3166
	38.9706	-107.0295	3372
Treasury Mountain	38.9188	-107.0365	2770
	38.9334	-107.0494	2832
	38.9537	-107.0614	2875
	38.9651	-107.0598	3073
	38.9707	-107.0587	3221
	38.9863	-107.0619	3394

Table 1: GPS coordinates of litter bag deployment sites

Discussion

The difference in fungal communities associated with live vs. dead plant matter is not surprising. Further analysis is needed to identify the species that were isolated, but a tentative hypothesis would be that there is a higher abundance of saprotrophic fungi in the litter cultures compared to the tiller cultures.

No data regarding the decomposition experiment have been gathered yet, given that it will require at least until the end of summer 2015 to yield useful results.

Once all facets of this study have been completed, we will be able to correlate associated fungal species from the plating project with decomposition rates to see if certain taxa or combinations of taxa have an effect on carbon cycling, and future

models will be able to incorporate these data to better understand overall ecosystem functioning in the context of shifting climates.

Plant-associated fungal endophytes play an important role both in the lives of their hosts and in the ecosystems of which they are a part. This study hopes to provide further information on these organisms of which relatively little is known.

In addition, the onset of global climate change threatens the stability of natural systems worldwide, understanding how decomposition might change as a result is therefore paramount to predicting overall ecosystem functioning in the future. This will allow for more informed conservation efforts and adaptive strategies in the future.

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Part II: How does endophyte symbiosis affect host survival and growth?

Introduction

Plant-symbiotic endophytic fungi are found almost everywhere on the planet, with a diversity rivaling that of insects (Caroll 1988; Arnold et al. 2000), and are estimated to inhabit 20-30% of all grass species (Leuchtmann 1992). These fungi provide various services to their hosts including protection from herbivory (Clay 1996), pathogen damage (Arnold et al. 2003), and abiotic stress (Malinowski & Belesky 2000; Song et al. 2012) in exchange for organic materials. Fungal symbioses may also be important in mitigating abiotic stresses caused by climate change (Marks & Clay 1996; Kivlin et al. 2013), as well as altering ecosystem processes and characteristics such as forest succession (Rudgers et al. 2007) and species diversity (Rudgers & Clay 2008).

Fungal endophytes can have diverse effects on the ecology of their host plants. For example, endophyte presence increased biomass production within *Phragmites australis* (Ernst et al. 2003). However, this effect may reverse depending on abiotic conditions such as water availability (Morse et al. 2002; Zhang and Nan 2007), and/or at what point in the plant's life cycle one takes measurements (Spiering et al. 2006). Another study found that endophyte presence decreased host plant biomass and survival ability of, but increased their reproductive capabilities (Rudgers et al. 2012). It has also been found that the number of plant-endophyte symbioses decrease with elevation (see figure 1).

We used a common garden experiment at the RMBL to test for effects of endophyte symbiosis on the survival and growth of a dominant, native host plant, *Festuca thurberi*. Because this experiment did not take place under water-limited conditions, we hypothesize that endophyte presence will have a negative effect on plant biomass and survival.

Methods

Common garden experiment. During summer 2011, individuals of *F. thurberi* from three different treatments were planted at the same site as our litter collections took place (38.96255014, -106.9852277; elevation 2992 m). These treatments included E+ plants, E- plants that had their endophyte removed experimentally using the fungicide Benomyl (EB-), and control E- plants (EC-) that were naturally endophyte-free. We used a series of ANCOVA analyses in SAS to compare survival rates, tiller and inflorescence counts, and height between E+ and E- plants, as well as between benomyl-treated E- and naturally E- individuals.

Seed collection. We collected seeds from naturally occurring plants at the site during September 2010.

Endophyte removal. Seeds slated for endophyte removal were placed in petri plates with Benomyl (2g/L) for 5 weeks at 4°C. Control seeds were placed on petri plates with water at 4C for the same time period. Plates were removed from the cold stratification and seedlings were allowed to germinate in the greenhouse.

Plant propagation. As seedlings germinated, we transferred them into plastic 12 packs filled with Pro-mix soil where they grew for 2 to 6 weeks. Then, plants were transplanted into 10 cm square pots filled with 1:1 mixture of Pro-mix and

play sand for 2 to 8 weeks before being re-potted into large pots (6.4 cm diameter, 35.6 cm deep), in which they grew for 4-6 weeks before transfer to the field. Pots were acclimated to field conditions for 1 week prior to transplanting into the common garden during which time leaves were misted daily to reduce the effects of change in humidity relative to the greenhouse.

Common garden design. On July 1, 2011, plants were planted in three rows and spaced 1 m apart. Plants were planted into the existing vegetation which included the native *F. thurberi* from which the seeds were collected. Each plant was randomly assigned to a location in the garden and tagged with a landscape staple and metal tag on the roadward side, and with the original plastic tag on the fenceward side. Plants were also marked with a colored stake flag. After transplanting, the plants were watered on July 1, July 3, July 5, July 21, July 23, and August 27 applying 1.89 L tap water per plant. No further manipulations were applied after these waterings.

Response variables. Initial tiller counts, influorescence counts, height, survival, and gopher disturbance data were collected upon transfer of plants into the garden on July 1, 2011, then retaken on August 23, 2012, August 31, 2013, and July 30, 2014. A series of two-way ANCOVAs were used to compare means of each variable between years.

Results

Survival rates. We found that endophyte-free plants had higher survival rates ($p=0.0009$) than endophyte-symbiotic plants, however, this effect did not change

significantly between years (Figure 4). The Benomyl treatment had no effect on survivability.

Tiller counts. The data suggest that there was a significant difference ($p=0.0012$) in average tiller counts per year between E+ and E- plants, E- plants have, on average, a higher number of tillers (Figure 5). Year and the interaction between year and endophyte status were not found to be significant factors, although in 2014, the trend was for higher tiller numbers in E+ compared to E- plants. There was no effect of benomyl on tiller counts of E- plants.

Inflorescence counts. We found no factors influencing inflorescence counts between E+ vs. E- treatments, nor benomyl vs. naturally E- treatments, but very few plants (2) have reproduced.

Plant height. There were no significant factors influencing plant height. However, year was nearly significant ($p=0.0861$) (Figure 6). Benomyl had no effect on height.

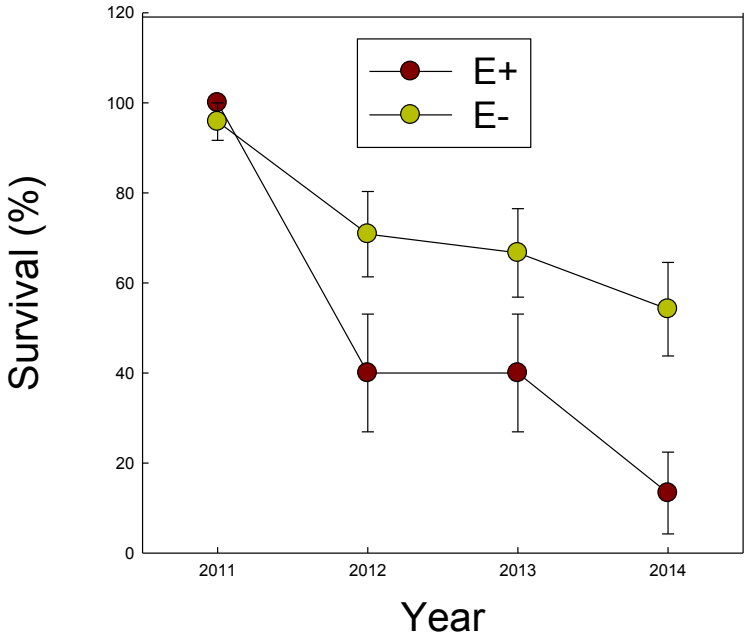

Figure 4: Survival rate comparison of E+ vs. E- plants 2011-2014

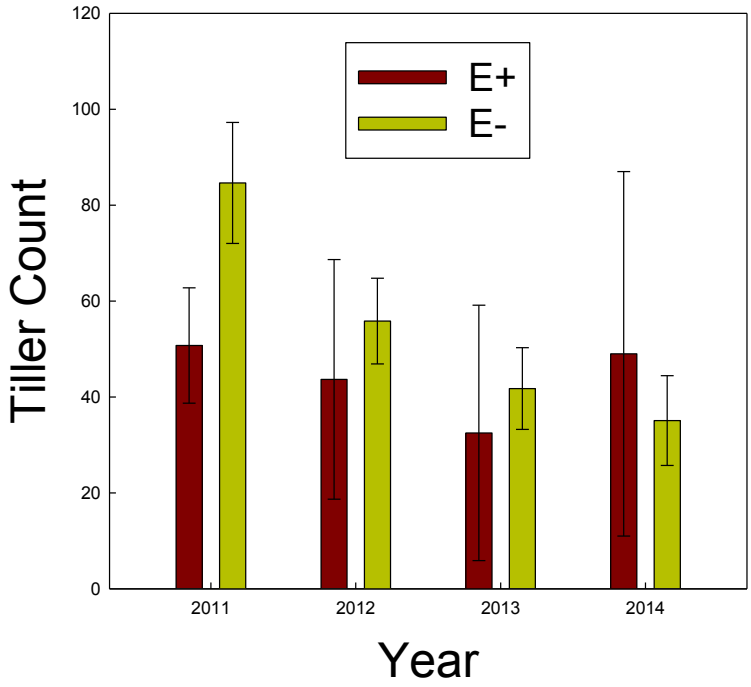

Figure 5: Tiller count comparison of E+ vs. E- plants 2011-2014

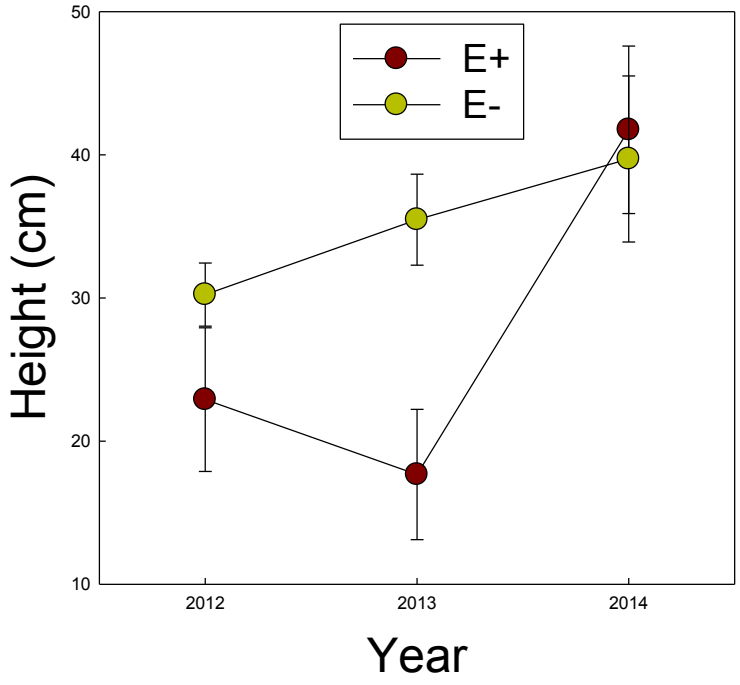

Figure 6: Height comparison of E+ vs. E- plants 2012-2014

Conclusion/Discussion II

Our results followed patterns discovered in past studies, indicating that endophyte presence may have an overall negative effect on survival and biomass of *Festuca thurberi*. This perceived detriment could possibly be mitigated if the endophyte were to enhance its host's reproductive abilities, as was shown by Rudgers et al. 2012. However, the possibility that the endophyte may have been behaving parasitically should not be discounted (Morse et al. 2002; Faeth & Sullivan 2003; Kogel et al. 2006). Further experimentation will be needed in order to determine the validity of either of these hypotheses.

This work helps to further elucidate the complex interactions between fungal endophytes and their plant hosts. These symbioses are dynamic and not always

mutualistic in nature. Gaining additional knowledge of how these two types of organisms interact with one another in the context of environmental conditions will provide ecologists with an enhanced understanding of how communities behave and why they are subject to certain changes and variations. Furthermore, a thorough understanding of community behavior will provide for more powerful analytical ecosystem models that will be useful for predicting and monitoring responses to climate change and other forms of disturbance.

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