EFFECTS OF *EPICHLOË COENOPHIALA*–TALL FESCUE SYMBIOSIS ON PLANT-MICROBE-SOIL INTERACTIONS IN A TEMPERATE PASTURE

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EFFECTS OF *EPICHLÖË COENOPHALA*–TALL FESCUE SYMBIOSIS ON PLANT-MICROBE-SOIL INTERACTIONS IN A TEMPERATE PASTURE

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DISSERTATION

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture, Food, and Environment at the University of Kentucky

By

Lindsey Christine Slaughter

Lexington, Kentucky

Director: Dr. Rebecca McCulley, Associate Professor of Plant and Soil Sciences

Lexington, Kentucky

2016

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ABSTRACT OF DISSERTATION

EFFECTS OF *EPICHLÖĘ COENOPHIŁA*–TALL FESCUE SYMBIOSIS ON PLANT-MICROBE-SOIL INTERACTIONS IN A TEMPERATE PASTURE

Plants interact in myriad ways with microorganisms to influence ecosystem processes such as nutrient cycling, which can regulate ecosystem response to global change. One important plant-microbe symbiosis occurs between cool-season grasses and asexual fungal *Epichloë* endophytes, such as tall fescue (*Schedonorus arundinaceus*) and *Epichloë coenophiala*. Because the common toxic strain of the endophyte (CTE) harms grazing livestock, non-livestock toxic endophyte (NTE) strains have been developed and are increasingly deployed in pastures. Little is known about how these symbioses impact other plant-microbe interactions and microbe-mediated soil processes in grassland ecosystems. I conducted three studies to determine how *E. coenophiala* presence (+) or absence (−) and differences in endophyte strain affected plant-microbe-soil interactions both within tall fescue and in surrounding plants. I hypothesized that presence of CTE in tall fescue (CTE+) would suppress presence and/or activity of other microbial symbionts and related processes compared to E− tall fescue, and NTE+ tall fescue effects would be intermediate.

My first field study examined how endophyte presence and strain in tall fescue influenced symbiotic biological nitrogen fixation (BNF) in red clover, biologically-fixed N uptake in tall fescue, and non-symbiotic BNF in soils. I found that tall fescue hosting different NTE+ strains utilized different amounts of biologically-fixed N. My second field study investigated how endophyte presence and strain impacted belowground mycorrhizal colonization within the same host plant. I found no significant differences in either AMF or dark septate endophyte (DSE) colonization in tall fescue in this study. In my third field study, I investigated how these belowground symbioses were potentially altered both by tall fescue-*E. coenophiala* genetics and future climate change. AMF functional structures such as arbuscules in roots and extraradical hyphae in soils were significantly affected by tall fescue genotype and endophyte status. I also found that some competitive symbiont interactions were ameliorated whereas others were exacerbated by future climate change conditions such as warming and added precipitation.

Overall, the results of these studies suggest that genetically distinct *E. coenophiala*-tall fescue associations, through alteration of plant-microbe-soil interactions,
will have divergent roles and long-term impacts on host-symbiont species interactions and nutrient cycling within pasture ecosystems.

**KEYWORDS**: arbuscular mycorrhizal fungi, biological nitrogen fixation, climate change, dark septate endophyte, grassland, *Neotyphodium*
EFFECTS OF *EPICHLOË COENOPHIALA*–TALL FESCUE SYMBIOSIS ON PLANT-MICROBE-SOIL INTERACTIONS IN A TEMPERATE PASTURE

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April 19, 2016
To my sisters, Jennifer and Leeann.
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Chapter One

Effects of plant-microbial symbiont interactions on soils: The *Epichloë coenophiala*-tall fescue symbiosis as a model system

1.1. Plant-microbial symbiont interactions

Interactions between plants and microorganisms are ubiquitous, occurring both within and on plant surfaces [e.g., (Carroll, 1988; Compant et al., 2010; Hirano and Upper, 2000; Partida-Martinez and Heil, 2011; Smith and Read, 2008)]. Plant-microbe interactions are often symbiotic, characterized by plant and microbial species living and functioning in close physical proximity (De Bary, 1879). Various bacterial and fungal symbionts may associate with either the aboveground or belowground portions (or both) of plant hosts, and their interactions vary from parasitic or pathogenic, which harm or impair plant function, to mutualistic, in which microorganisms provide beneficial services for the host plant, such as enhanced nutrient availability and increased productivity [(Fig. 1.1; (Bronstein, 1994; Johnson et al., 1997)].

When residing asymptotically within plant tissues for all or part of their life cycles, both bacterial and fungal plant symbionts are specifically known as “endophytes” (Wilson, 1995). Although the function of many plant endophytes is not well-known, there are two key plant-endophyte nutrient transfer symbioses that are well-studied: the legume-rhizobia and plant-mycorrhizal interactions. Though these symbionts are ‘endophytes’ by literal definition, they tend to be excluded from the general use of the term when engaged in nutrient transfer symbioses. This is in part to clarify their important functional role as intracellular symbionts that form specialized nutrient-transfer structures within host plants.
Rhizobia and mycorrhiza symbionts are first attracted to plant roots by plant secondary chemical exudates such as flavonoids and strigolactones in the rhizosphere (Abdel-Lateif et al., 2012; Steinkellner et al., 2007). Initiation of bacterial rhizobium symbiosis and mycorrhizal colonization such as by arbuscular mycorrhizal fungi (AMF) then begins with signaling molecules produced by the bacteria and fungi which diffuse to the host plant, referred to respectively as Nod- and Myc factors, (Dénarié et al., 1996; Schmitz and Harrison, 2014). Some Myc factor molecules are structurally similar to Nod factors, such as lipo-chito-oligosaccharides (Maillet et al., 2011), suggesting that parts of the plant-symbiont signaling pathways during infection are very similar between these two nutritional symbionts. In fact, many essential plant genes required for proteins used in signal transduction and formation of both rhizobium and AMF associations are shared, albeit with potentially different rhizobium- or AMF-specific biochemical interactions during the infection process (Genre and Russo, 2016). What ultimately results, however, are two types of specialized plant-symbiont nutrient transfer interfaces: visible swellings on plant roots (nodules) that harbor communities of nitrogen-fixing rhizobia (Ferguson et al., 2010), and specialized finely-branched intracellular AMF hyphal clusters, or arbuscules (Carling and Brown, 1982). These structures are the sites of nutrient exchange between plant and symbiont, whereby the rhizobia or AMF symbiont is sustained through plant photosynthetically-produced C in exchange for vital nutrients such as atmospherically-derived N from rhizobia (Ferguson et al., 2010) or inorganic phosphate from AMF (Cox and Tinker, 1976; Smith and Read, 2008).

Other plant symbionts, including endophytes, frequently exist alongside these two nutritional symbionts. Some of these asymptomatically impart nutritional benefits to their
Plant hosts, such as endophytic N₂-fixing bacteria in stems and leaves (Dobereiner, 1992; Moyes et al., 2016). In addition, legume nodules formed by rhizobia symbionts frequently host various non-rhizobial bacterial endophytes. These endophytes lack specialized nodule-formation genes (\textit{nodC}) and nitrogen-fixation genes (\textit{nifH}), yet reside in nodules formed by rhizobia symbionts (De Meyer et al., 2015; Peix et al., 2012). Mycorrhizae, including AMF, also host their own microbiome of bacterial symbionts within and on fungal structures, such as hyphae and spores, further complicating the network of nutritional interactions within and around host plants (Bonfante and Anca, 2009; Desirò et al., 2014; Salvioli et al., 2016).

Plant-associated symbionts, including endophytes, are also functionally important in determining host plant responses to environmental factors, such as serving as an epigenetic means of plant adaptation to habitat-specific stresses such as heat, drought, and salt tolerance (Coleman-Derr and Tringe, 2014; Rodriguez et al., 2008). For example, a fungal endophyte \textit{Curvularia sp.} has been shown to increase thermotolerance of panic grass (\textit{D. lanuginosum}) in soils above 40 °C (Redman et al., 2002). In addition, association of cheatgrass with thermotolerant fungal endophytes such as \textit{Morchella sp.} contributes both to increased plant growth and fecundity and to survival after fire events, thereby enhancing fire-adaptation and invasive potential of the grass host (Baynes et al., 2012). In drought conditions, AMF species isolated from soils adapted to water limitation can improve plant growth, especially in conjunction with similarly adapted rhizosphere bacteria (Marulanda et al., 2009). Colonization by AMF can also alleviate the effects of soil salinity and increase plant growth, especially when AMF strains are well-adapted to saline or salt-stressed environments (Dashtebani et al., 2014; Hajiboland, 2013). How
these environmental stress tolerance responses are constructed likely vary genetically and biochemically, and the complex communication pathways that regulate these responses are often not well known. Yet, they are generally thought to include mechanisms such as symbiont regulation of plant water use and efficiency or accumulation of reactive oxygen species (Rodriguez et al., 2008).

Plant symbionts and endophytes can also influence above- and belowground herbivory, herbivore/pathogen community composition, and associated trophic dynamics, sometimes through the production of secondary metabolites (Gunatilaka, 2006; Schardl et al., 2007; Tan and Zou, 2001). For example, aboveground fungal endophytes of the genus *Epichloë* are well-known to deter both above- and belowground herbivory in cool-season grass hosts by producing mammal- and insect-toxic alkaloids (Clay, 1988). However, *Epichloë uncinata* (W. Gams, Petrini & D. Schmidt) [= *Neotyphodium uncinatum* (W. Gams, Petrini & D. Schmidt)] deters belowground feeding by grass grub larvae by decreasing root volatile emissions (Rostas et al., 2015), in addition to translocating loline alkaloids to root tissue (Patchett et al., 2011), suggesting that these endophytes can employ many host-protective mechanisms.

Indirectly, AMF can also contribute to host protection from herbivores through improved plant growth and nutrient status. For example, milkweed inoculated with AMF regrew more quickly after monarch caterpillar herbivory and produced more chemical defense compounds due to enhanced foliar N and P concentrations, growth rate, and root biomass (Tao et al., 2016). However, this effect was not observed in soybean plants inoculated with AMF and subjected to herbivory by Mexican bean beetle, despite AMF-related alleviation of phosphorus deficiency and improved plant growth (Borowicz,
Inoculation with *Rhizobium leguminosarum* symbionts decreases broomrape parasite damage in pea plants by increasing lignin and phenolic compound concentrations (Mabrouk et al., 2010), and to protect lentil plants from *Fusarium oxysporum* pathogen infection (Essalmani and Lahlou, 2003). Many bacterial endophytes deter bacterial and fungal pathogens in agricultural crop species, in addition to promoting plant growth, through potential mechanisms such as increased mineral uptake or suppression of unfavorable microbes (Hallmann et al., 1997).

The role of some plant symbionts and endophytes can, pleiotropically, alternate between parasitic and mutualistic depending on numerous biological and environmental factors that influence the relative benefits to both host and symbiont. A symbiont’s role can change depending on its location within or on the host plant, the developmental stage or life phase of both symbiont and host, and influences by external biotic and abiotic conditions (Johnson et al., 1997; Müller and Krauss, 2005; Newton et al., 2010; Saikkonen et al., 1998; Schulz and Boyle, 2005). For example, endophytic interactions in putative hosts are presumably more likely to be mutualistic when in roots than in aboveground organs, both because roots are the plant C sink in closest proximity to minerals, water, and microbially-degraded nutrients that are available for trade, and because roots are less physically limiting to symbiont colonization through lack of protective tissue structures like waxy epidermal cuticles (Schulz and Boyle, 2005).

Symbionts can also alternate between asymptomatic endophytes and other roles like mutualists or pathogens depending on life stage and infection strategy. Mycorrhizae can function as endophytes rather than nutritional symbionts (distinguished by lack of arbuscules and beneficial nutrient transfer) when colonizing non-mycorrhizal host plants.
or non-root plant tissues, such as rhizome scales, or during extended life phases such as long-term persistence in older host roots (Brundrett, 2004). Newton et al. (2010) point out that many well-known plant pathogens include asymptomatic life cycle stages, which can be quite long or occur multiple times, before or after active pathogenic damage to their host plants, sometimes even existing as beneficial symbionts in the interim.

External biotic and abiotic factors at multiple ecosystem scales heavily influence the degree of beneficial or detrimental symbiont interactions with hosts [Fig. 1.2; (Johnson et al., 1997)]. For example, in preferentially shade-growing common tropical palm, the fungus Diplodia mutila commonly exists as an asymptomatic endophyte, yet it pathogenically infects seedlings under high light conditions (Álvarez-Loayza et al., 2011). Two more widely-studied fungal symbioses that frequently exhibit a continuum of beneficial to parasitic interactions within grass hosts are belowground fungi inhabiting plant roots, such as AMF and dark septate endophytes (DSE), and Clavicipitaceous endophytic fungi of the genus Epichloë inhabiting aboveground plant tissues (Johnson et al., 1997; Müller and Krauss, 2005). Mycorrhizae, for example, can potentially become parasitic towards their host with increasing soil fertility, as nutritive benefits of the symbiosis to the plant declines (Neuhauser and Fargione, 2004). Currently, we can predict the nature of an AMF association based on availability of vital nutrients like N and P, which are commonly acquired by AMF in exchange for plant C [Fig. 1.3; (Johnson, 2010)]. This effect, in which increased nutrient availability to plants generally decreases the benefit of maintaining a heterotrophic nutritional symbiont, thus destabilizing the mutualism, has been further demonstrated in other well-known associations such as algae–cyanobacteria and plant–rhizobia bacteria (Shantz et al.,
2016). With closer examination and characterization of a wider range of host-symbiont interactions in asexual *Epichloë* endophytes, construction of similar trade-balance models may be possible for these organisms. However, many researchers concur that while we have identified many of the ecological factors and scales that influence the functioning of plant-symbiont interactions along the continuum of associations, the mechanistic nature, relative importance, and complex interactive influences of these factors remain largely unknown across many plant-symbiont associations and situations [e.g., (Johnson et al., 1997; Müller and Krauss, 2005; Newton et al., 2010; Shantz et al., 2016)]. In addition, multiple fungal groups or species often infect the same plant host and may have complex interactions that affect the host, the above- and belowground communities, and resulting ecosystem processes.

### 1.2. Plant-microbe effects on the soil ecosystem

In soil, many of the microbes that interact with plants in the rhizosphere are free-living (non-symbiotic) organisms. For example some N$_2$-fixing bacteria species live in soil without forming rhizobia-like symbiotic or endophytic associations with plants (Kennedy et al., 2004; Paul and Newton, 1961). Plant-derived C and N are constantly flowing between plant roots and the soil environment during rhizodeposition of plant root cell material and exudates (Jones et al., 2009). Free-living microorganisms receiving these deposits often benefit plants, such as through increased rates of nutrient turnover and availability (Lambers et al., 2009). Yet, soil and rhizosphere communities are also the source of many plant symbionts, including endophytes (Compant et al., 2010; Lugtenberg and Kamilova, 2009; Rosenblueth and Martínez-Romero, 2006). The
complex interactions between plants and microbial symbionts can greatly influence soil ecosystem characteristics and processes.

Some well-studied nutritional symbionts, such as rhizobia and AMF, are frequently cited for their impacts on soil C and N cycling. Biological N₂ fixation, from both natural and agricultural uses of rhizobia-legume symbioses, is a significant terrestrial input in the global N cycle (Canfield et al., 2010; Herridge et al., 2008). The abundance of AMF, meanwhile, is often proportionally linked to ecosystem C storage and soil aggregate stability, often via binding of soil aggregates by AMF hyphae and exudates (Bronick and Lal, 2005; Jastrow et al., 1998; Wilson et al., 2009). Even symbioses between grasses and asexual *Epichloë* endophytes have been linked to accumulation of soil C and N, potentially due to endophyte-induced changes to soil microbial activity (Franzluebbers et al., 1999). Other soil properties such as soil pH can be affected by rhizobium-legume symbioses. For example, release of H⁺ during N₂-fixation in nodules is a rhizosphere-acidifying process (Dakora and Phillips, 2002; Maltais-Landry, 2015; Raven et al., 1990; Williams, 1980). Further, the composition of soil microbial communities can be affected by presence of AMF (Vestergård et al., 2008), and interactions between plant host, AMF, and free-living microbes can differentially impact organic matter decomposition, perhaps through mechanisms such as mycorrhizal suppression of microbial growth or activity (Moore et al., 2015).

Through these effects, plant-symbiont interactions are also major drivers of plant-soil feedbacks. Different plant species, regardless of symbionts, can influence the types and activities of soil microorganisms within communities (Berg and Smalla, 2009), but the structure and function of plant communities can also be shaped by soil microbes [e.g.,
Further, presence and diversity of plant symbionts such as *Epichloë* endophytes (Orr et al., 2005; Rudgers and Clay, 2007) and AMF (Bever et al., 2010; van der Heijden et al., 1998) can impact aboveground plant diversity and community composition. Through these broader ecosystem feedbacks and various plant-microbe interactions, associations between plants and microorganisms are often important determinants of terrestrial C [e.g., (Moore et al., 2015)] and N-cycling processes [e.g., (van der Heijden et al., 2008)]. Plant-microbe interactions can, therefore, be key drivers of soil ecosystem response to global change factors, such as eutrophication and climate change.

While plants and their associated microorganisms are considered “ecosystem engineers” (Jones et al., 1996; Lambers et al., 2009), our knowledge of plant-symbiont effects on soil ecosystems is limited by the lack of long-term, in situ studies that experimentally manipulate plant- or soil-microbial communities. Yet, much work suggests that economic, agricultural, and environmental gains can be made by more effectively utilizing plant- and soil-associated microorganisms and their interactions for future sustainability of natural and agricultural ecosystems. For example, many species of soil-borne bacteria existing in the rhizosphere and within or on plant roots have demonstrated beneficial effects on plant growth through various mechanisms, thus earning the designation plant growth-promoting rhizobacteria [PGPR; (Kloepper and Schroth, 1978)], or plant growth-promoting bacteria [PGPB; (Bashan and Holguin, 1998)]. There is great interest in applying PGPR and PGPB species as commercial inoculants to agricultural plant species to increase plant growth and tolerance to biotic and abiotic stresses, yet the effectiveness of these applications in field studies is still...
largely unsuccessful and poorly understood (Compant et al., 2010; Lugtenberg and Kamilova, 2009). In addition, the long term effects of introducing PGPR and PGPB species into agricultural ecosystems are yet undetermined (Ambrosini et al., 2015). Considering the complex yet variable relationships between plants and microbes, far more research is required to effectively predict long-term ecosystem responses to manipulations of plant- and soil-microorganismal interactions.

1.3. Challenges to assessing the role of microbes in plant-soil interactions

Quantifying the contribution of specific microorganisms to plant-soil interactions and soil processes is often problematic given the myriad, uncontrollable factors that complicate conclusions from field studies, such as opportunistic herbivory, spatial/temporal influences on microbial communities, and competition between native and introduced species. For example, field studies of added soil biota may be subject to grazing by non-manipulated soil fauna, such as springtails, which can alter interactions between AMF and the soil microbial community and reduce AMF infection potential (Caravaca and Ruess, 2014). Once applied, beneficial microbes may not persist in the soil community long-term. For example, levels of inoculated *Sinorhizobium meliloti* have been shown to become undetectable in as little as 32 d in a nutrient-poor soil, and only 23% of the wild-type strain applied could be recovered after 64 d in a nutrient-rich soil (Da and Deng, 2003).

When microbes introduced to crops persist through the growing season, their numbers may steadily decline with time relative to native microbes [e.g., Table 3 in Schippers et al. (1987)]. Successful inoculation and persistence of bacterial symbionts, such as rhizobia for legume nodulation, is often attempted via the application of seed
coatings, but the efficacy of this technique is still a common concern despite substantial advancements in technology and knowledge of factors affecting bacterial survival on seeds (Deaker et al., 2004). Inoculated symbionts may be reduced through competition or other interactions with indigenous microorganisms [e.g., (Abbott and Robson, 1982; Hepper et al., 1988; Thies et al., 1991)]. Use of commercial AMF inoculum, for example, is generally less effective or persistent than using whole inoculum obtained from a similar or reference ecosystem, likely due to complementary host-symbiont interactions enabled by previous co-habitation in a similar environment (Maltz and Treseder, 2015). Competition between isolates after application of mixed species inoculum may also reduce their effectiveness (Mickelson and Kaeppler, 2005). Therefore, manipulating plant and microbial communities in long-term ecosystem studies can be problematic to effectively implement and maintain.

One relatively easily-manipulated plant–microbe interaction is that of grasses and their aboveground fungal endophytes of the genus *Epichloë*. For example, many cool-season grasses form host-specific associations with asexual *Epichloë* fungal endophytes within aboveground tissues (Carroll, 1988). These fungal endophytes, when functioning as mutualists, often produce secondary metabolites (alkaloids) that deter mammalian or insect herbivory of their hosts (Clay, 1988), with a naturally wide genetic variety of fungal strains able to produce different amounts and types of these compounds (Schardl et al., 2013b; Takach et al., 2012; Takach and Young, 2014).

For a few agronomically important grasses, such as tall fescue (*Schedonorus arundinaceus* Schreb. Dumort) and perennial ryegrass (*Lolium perenne* L.), the presence and strain of their epichloïd symbionts have been manipulated within improved cultivars
of these grasses (Bouton et al., 2002; Latch and Christensen, 1985; West et al., 1998).
Because these asexual epichloid symbionts are vertically transmitted, albeit sometimes imperfectly (Afkhami and Rudgers, 2008), and systemically inhabit plants throughout their life cycle, compatible endophytes inserted into grass cultivars are naturally transmitted and persistent in subsequent plant generations. Although it is theoretically possible for two or more asexual *Epichloë* species to co-infect and hybridize within host plants [e.g., (Groppe et al., 1995; Tsai et al., 1994)], coinfection by multiple species is hard to achieve in laboratory manipulations and rarely persists in nature (Christensen et al., 2000; Wille et al., 1999).

The long-term, constitutive nature of these symbioses makes *Epichloë*-grass models extremely useful for ecosystem studies of plant-microbe interactions and their effects on ecological processes. In addition, genetic characterization and analysis of alkaloid production pathways allows researchers to distinguish between different *Epichloë* strains within individual grasses (Takah et al., 2012). Manipulation of *Epichloë* presence and strain within cool-season grasses is therefore a viable and controllable model system for studying the role of microbes in plant-microbe-soil interactions.

1.4. *Epichloë coenophiala*-tall fescue symbiosis

Fungal endophytes of the family Clavicipitaceae, including those of the genus *Epichloë*, commonly associate with cool season grasses of the family Poaceae, subfamily Poëideae (Clay and Schardl, 2002; Schardl et al., 2004; White Jr., 1987). These Clavicipitaceous endophytes may have originated from pathogens of animals, such as arthropods, that jumped to grass hosts (Spatafora et al., 2007). With time, these endophytes, particularly those of the genus *Epichloë*, coevolved with their grass hosts
such that many *Epichloë* species today can associate specifically with a single grass species (Ekanayake et al., 2012; Schardl et al., 2004). The evolutionary and ecological mechanisms by which *Epichloë* endophytes have coevolved with Poöideae grasses have been the subject of multiple detailed reviews (Clay and Schardl, 2002; Schardl, 2010; Schardl et al., 2004).

Systemic and asymptomatic colonization within aboveground portions of grass tissue by asexual *Epichloë* species, which may only propagate vertically through dissemination in seeds, have come to play a constitutive role in host grass evolution and ecological interactions. In certain environments and hosts, some asexual *Epichloë* endophytes produce commensal or antagonistic effects on hosts (Faeth, 2002; Saikkonen et al., 1998). Yet, the ability of asexual *Epichloë* endophytes to produce a suite of protective neurotoxic alkaloid compounds to defend their host against herbivory frequently contributes to a mutualistic relationship between plant and symbiont (Bush et al., 1997; Clay, 1988).

These alkaloid compounds, such as the mammal-toxic ergot and indole diterpene alkaloids, and the insect-toxic loline and peramine alkaloids, are produced at diverse biochemical levels, depending in large part on genetic capability such as by endophyte strain (Scharld et al., 2013a; Scharld et al., 2013b; Scharld et al., 2013c). The most common *Epichloë* strains associating with agronomically-important grasses such as tall fescue and perennial ryegrass produce toxic alkaloids to deter mammalian herbivory, such as ergovaline in tall fescue and lolitrem B in ryegrass. Yet, due to well-reviewed toxicity symptoms in livestock consuming these grasses as forage and subsequent losses to the animal production industry (Hoveland, 1993; Klotz, 2015; Strickland et al., 2011),
much effort has been made to manipulate *Epichloë*-grass combinations, such as that between *E. coenophiala* and tall fescue, a cool-season grass that is native to continental Europe and the Mediterranean Basin (Borrill, 1972; Borrill et al., 1971), to retain many of the mutualistic benefits of endophyte infection while reducing production of alkaloids that are toxic to livestock. Endophyte strains in these associations are referred to as ‘non-toxic’ or ‘novel’ endophytes (NTE), while the more commonly distributed *E. coenophiala* strains which produce livestock-toxic alkaloids are referred to as ‘common toxic’ endophyte (CTE).

It is possible to select for combined endophyte and grass characteristics during traditional breeding and backcrossing, as was unknowingly accomplished during development of early cultivars such as ‘Kentucky 31’ and ‘Kenhy’ tall fescue, which hosted CTE strains (Bacon et al., 1977; Fergus and Buckner, 1972; Siegel et al., 1984). Developing tall fescue cultivars that host NTE strains via traditional backcrossing and selection is unfeasibly complex and time-consuming. The preferred method for manipulating endophyte presence and strain within selected tall fescue cultivars instead involves isolating desirable endophytes, such as NTE strains that naturally do not produce livestock-toxic alkaloids, in pure culture and inserting them directly into tall fescue lines (Latch and Christensen, 1985). More recently, researchers have also successfully experimented with using genetic modification to directly control expression of alkaloid biosynthesis genes in asexual *Epichloë* strains, such as to disable production of the livestock-toxic alkaloid ergovaline (Panaccione et al., 2001). The ability to manipulate plant and symbiont genetics allows researchers to investigate the roles, mechanisms, and effects of these plant-microbe associations on various ecological scales.
1.5. Role of endophyte symbiosis in tall fescue physiology and ecology

Symbiosis with *Epichloë* sp. can result in various on grass hosts, from parasitic or antagonistic to mutualistic, depending on factors such as host and symbiont genetic backgrounds, both biotic and abiotic interactions (Müller and Krauss, 2005). A large proportion of studies, usually conducted in agronomic pasture ecosystems in which tall fescue is not a native plant, suggest that the relationship between *E. coenophiala* and tall fescue is generally commensalistic or that of a defensive mutualism, whereby the intercellular endophyte receives nutrients and reproductive transmission from tall fescue in exchange primarily for producing protective alkaloids to deter herbivory (Clay, 1988). The types and production levels of these alkaloids varies according to endophyte and host genetics and spatial or temporal variation in controlling environmental parameters. CTE strains of *E. coenophiala* infecting tall fescue in North America produce mammal-toxic ergot alkaloids in addition to insect-toxic loline and peramine alkaloids, while strains classified as NTE do not produce mammal-toxic alkaloids (West et al., 1998).

Plant and endophyte genotypes further regulate alkaloid production levels (Agee and Hill, 1994; Roylance et al., 1994). The concentration of some alkaloids can vary seasonally with preferential location in different plant parts, such as ergovaline peaking during seed head production in tall fescue (Agee and Hill, 1994; Rottinghaus et al., 1991). Loline alkaloids can also be found in highest concentrations in the seed, and generally increase with plant age (Bush et al., 1993). Loline alkaloids may be found in low concentrations in the roots, and both root and shoot concentrations increase in response to drought stress (Nagabhyru et al., 2013). Alterations in climate such as warming increase ergot alkaloid concentrations in tall fescue (Bourguignon et al., 2015;
McCulley et al., 2014), while growth and nutrient-altering pressures such as frequent defoliation can reduce ergot alkaloid levels (Belesky and Hill, 1997; Salminen and Grewal, 2002). Each of these factors can interactively contribute to the degree of herbivore protection conferred to tall fescue.

Most studies conducted regarding mammalian-herbivore deterrence (primarily fescue toxicosis due to ergot alkaloids) in *E. coenophiala*-infected tall fescue are related to livestock performance and productivity. There are a few reports of CTE+ tall fescue effects on non-livestock mammals. Tall fescue consumption has raised concerns about survival and reproductive performance in wild rabbit populations (Giuliano et al., 1994). Further, increased abdominal lipomatosis due to consumption of tall fescue has been documented in Eld’s deer (Wolfe et al., 1998). Populations of prairie voles grazing on CTE+ tall fescue plots exhibited greater weights at sexual maturity, perhaps due to delayed onset of sexual maturity, yet endophyte infection did not affect reproduction (Fortier et al., 2000). Feeding CTE+ tall fescue to meadow voles also did not affect reproduction, body temperatures, or mortality rates in Conover (1998), yet mortality significantly increased in CTE+ tall fescue-fed voles when ambient temperatures increased to 31 °C. Overall, it seems that many wildlife species such as birds and small mammals prefer not to eat CTE+ tall fescue leaves or seeds, but feeding trials produce few endophyte-related consequences for growth or reproduction (Barnes et al., 2013).

In addition, multiple mechanisms other than alkaloids have been shown to contribute to herbivore defense, such as increased silicon content and other secondary metabolites, in related grass species such as meadow fescue (Huitu et al., 2014). Although studies have investigated the moderate benefits to livestock species such as
cattle and lamb consuming NTE+ tall fescue compared to E−, or CTE+ tall fescue, [e.g., (Drewnoski et al., 2009; Franzluebbers et al., 2009; Parish et al., 2013)], I found no studies to date investigating the effects of NTE+ tall fescue on non-livestock mammalian herbivore populations.

The deterrent effects of CTE+ tall fescue on herbivory by certain insects are also well documented, and often related to production of loline or peramine alkaloids that are found in CTE and NTE strains [e.g., (Breen, 1994; Clay et al., 1993; Davidson and Potter, 1995; Rudgers and Clay, 2008)]. Yet, alkaloid production does not appear to be wholly responsible for negative effects of tall fescue consumption on insects. For example, CTE+ tall fescue fed to grass skipper butterfly larvae did not impair growth or survival compared to E− tall fescue, but neither tall fescue treatment performed as well as Kentucky bluegrass (Jokela et al., 2016). In addition, studies comparing effects of CTE+ and NTE+ tall fescue, both of which should produce insect-deterring alkaloids, albeit at varying levels, have reported mixed results. Pastures containing NTE+ tall fescue had similar numbers of chewing insects (grasshoppers, crickets, caterpillars) or sucking insects (leafhoppers or planthoppers) to pastures containing CTE+ tall fescue (Keathley and Potter, 2012). Tall fescue harboring the NTE strain AR542 exhibited moderate levels of aphid population growth compared to E− (least resistant) and CTE+ (most resistant) tall fescue (Hunt and Newman, 2005). Even different tall fescue cultivars harboring the same NTE strain (AR542) produced differing effects on invertebrate communities (Yurkonis et al., 2014), suggesting that unique host-symbiont genetic combinations will differently impact ecosystem dynamics affected by insect herbivores. Because herbivore-deterrence is a strong contribution to mutualism between E. coenophiala and tall fescue
[e.g., (Rudgers and Clay, 2007)], future research should investigate the genetic, biotic, and abiotic controls on herbivore interactions with tall fescue and their resulting ecosystem effects, especially with regard to the increasing presence of NTE+ tall fescue associations.

While increased tolerance or resistance to herbivory frequently influences the degree of mutualism between \textit{E. coenophiala} and tall fescue, this symbiosis also impacts plant characteristics like drought and mineral stress tolerance. Increased drought tolerance is perhaps mediated in E+ plants by antioxidants (Malinowski and Belesky, 2006), such as production of phenolic compounds in E+ plants that help protect cells from oxidative stress (Malinowski et al., 1998), or by endophyte-induced accumulation of other metabolites such as free neutral sugar or sugar alcohol compounds to aid in osmotic adjustment (Nagabhyru et al., 2013). CTE+ tall fescue has demonstrated higher survival than E− plants in greenhouse drought stress, in addition to greater regrowth after drought alleviation (Arachevaleta et al., 1989). Similarly increased CTE+ tall fescue survival under greenhouse drought stress may have been due to endophyte regulation of stomatal conductance (Elmi and West, 1995). Especially in course and medium-textured soils, increased drought tolerance in CTE+ tall fescue may be due to altered soil water release paths and increased plant available water (Hosseini et al., 2016). Yet, Elbersen and West (1996) found no consistent endophyte-related mediation of drought stress within three tall fescue genotypes apart from greater water retention in leaf sheaths. Assuero et al. (2000) observed that cultivar and endophyte status impacted tall fescue response to drought. For example, an E+ tall fescue cultivar of Mediterranean origin had less drought tolerance than one of temperate origin, while each received different benefits from different
endophyte associations. Hill et al. (1996) further found that in combinations of three endophyte and plant genotypes, only one resulted in endophyte-conferred adaptations to drought and prolonged moisture stress. In a study assessing the response of two CTE+ and two NTE+ tall fescue genotypes to warming and added growing season precipitation, Bourguignon et al. (2015) found that in only two of the genotypes (one CTE and one NTE) did endophyte symbiosis increase biomass production under warming and enhance overall drought recovery. These studies highlight how endophyte-conferred responses to environmental stress can vary widely depending on specific host-symbiont genetic combinations.

*Epichloë coenophiala* symbiosis in tall fescue has also been associated with alterations in nutrient efficiency and mineral acquisition. For example, tall fescue receiving N fertilization has exhibited greater biomass production in CTE+ than E− plants (Arachevaleta et al., 1989), which is potentially explained by greater activity of enzymes related to N-metabolism such as glutamine synthetase (Lyons et al., 1990). However, Rogers et al. (2011) found decreased N in E+ tall fescue compared to E− in the field, which was further influenced by interactive effects of plant and endophyte genotype. CTE+ tall fescue can also respond to P deficiency through mechanisms such as altering root morphology (Malinowski et al., 1999) and exudation of phenolic-like compounds to increase P-uptake and availability (Malinowski and Belesky, 2000), though these effects are inconsistent between genotypic host-symbiont combinations (Malinowski and Belesky, 1999). Few studies have considered NTE+ tall fescue genotypes. Some of these, such as AR542, may also produce phenolic-like compounds that bind Cu$^{2+}$ in response to P-deficiency (Malinowski et al., 2004). In P limitation,
symbiosis with either CTE or two NTE strains (AR542, AR584) produced greater plant dry matter yield of both roots and shoots compared to E− tall fescue (Ding et al., 2015b). Yet, symbiosis with these strains in another study by Ding et al. (2015a) produced no benefits to plant biomass or P-uptake from different P sources compared to E− tall fescue, despite significant decreases in the soil NaOH-P; fraction associated with AR542 compared to E−, and significant interactive effects of endophyte and P-source on potential acid phosphatase activity. Despite commonly cited endophyte-mediated enhancements to moisture and mineral stress tolerance, these effects clearly vary among environmental conditions and unique host-symbiont genotypic combinations.

In addition to, and potentially as a result of, the endophyte-related effects on herbivory, drought tolerance, and nutrient status in tall fescue, CTE+ tall fescue exhibits enhanced characteristics contributing to increased overall fitness. For example, Clay (1990) reported greater biomass, tiller production, flowering frequency, and plant survival in CTE+ tall fescue compared to E− grown over three years. CTE+ plants have been shown to flower earlier than E− plants (Newman et al., 2003), and to produce more seeds per plant and per panicle (Rice et al., 1990). At high ambient temperatures (approximately > 35 °C), CTE symbiosis can increase photosynthetic rates in tall fescue (Marks and Clay, 1996; Newman et al., 2003). CTE+ tall fescue can have larger root systems than E− plants (De Battista et al., 1990), which may contribute to drought tolerance effects discussed earlier. The interactive consequences of each of these endophyte-conferred benefits often cause CTE+ effects on composition and function of above and belowground plant communities.
In aboveground plant communities, increased competitive ability of CTE+ tall fescue can reduce diversity of surrounding plant species and increases in CTE+ tall fescue abundance with time (Clay and Holah, 1999). Endophyte symbiosis within invasive tall fescue can further disrupt correlations between plant diversity and ecosystem function such as primary productivity (Rudgers et al., 2004). Such competitive interactions between tall fescue and other plant species are often driven by endophyte-mediated factors such as alterations in herbivory (Clay et al., 1993; Rudgers et al., 2010). In addition, CTE+ tall fescue tissue leachate can reduce tree seedling emergence, suggesting that allelopathy via endophyte-produced secondary chemicals may play a role in tall fescue interactions (Orr et al., 2005). When considering NTE strains, field plots containing either CTE+ or NTE+ tall fescue can support significantly greater tall fescue abundance than in E− plots (Bouton et al., 2002; Iqbal et al., 2013). As repeatedly noted, though, specific host-symbiont genetic combinations often produce different plant community effects. Although plots containing NTE+ tall fescue housed 10% more plant species than similar plots containing CTE+ tall fescue, different effects on tall fescue abundance and endophyte infection frequency with time were observed between tall fescue cultivars hosting the same AR524 NTE+ strain (Rudgers et al., 2010). Yurkonis et al. (2014) found that AR542 NTE symbiosis significantly reduced tall fescue abundance relative to CTE symbiosis in one tall fescue cultivar, and reduced species evenness of the aboveground plant community relative to CTE symbiosis in another cultivar.

1.6. Belowground effects of endophyte symbiosis

Despite the widespread distribution and use of tall fescue in agroecosystems and documented impacts on aboveground plant and animal communities, relatively few
studies have investigated the belowground effects of aboveground *E. coenophiala* symbiosis. Yet, aboveground *E. coenophiala* symbiosis in tall fescue produces a range of positive, negative, neutral, or equivocal effects on belowground properties such as soil nutrient cycling and soil macro- and microfauna communities [reviewed in (Omacini et al., 2012; Rudgers and Clay, 2007); also see Table 1 in McNear and McCulley (2012)]. For example, in greenhouse pot experiments, CTE+ tall fescue completely repressed numbers of an inoculated root-knot nematode (Elmi et al., 2000), yet had no effect on numbers of a different parasitic nematode species in Kimmons et al. (1990). In assays using extracts from CTE+ tall fescue roots, the loline alkaloid N-formylloline attracted root parasitic nematodes at low concentrations yet repelled them at high concentrations, while all levels of the ergot alkaloid ergovaline repelled nematodes and induced mortality (Bacetty et al., 2009). Earthworms have exhibited increased growth when fed only CTE+ tall fescue leaf tissue compared to E− (Humphries et al., 2001), yet no effect on earthworm abundance was observed between field plots of CTE+ and E− tall fescue (Davidson and Potter, 1995). In addition, while abundance of key springtail detritivores were unaffected by CTE+ tall fescue, community composition of springtail species diverged in response to aboveground endophyte infection (Lemons et al., 2005).

Perhaps related to the effects on soil fauna, grasslands dominated by CTE+ tall fescue commonly exhibit increased C and N sequestration compared to E− tall fescue [e.g., (Franzluebbers and Hill, 2005; Franzluebbers and Stuedemann, 2002, 2005; Iqbal et al., 2012)]. This may also be related to endophyte-mediated effects on soil microbial communities and activity, although literature findings are mixed. CTE+ tall fescue can increase field soil microbial biomass C (Handayani et al., 2011) and soil microbial lipid
biomass (Iqbal et al., 2012) compared to E− tall fescue fields. Plant-free soils receiving rhizodeposit solutions collected from aseptic microlysimeter units growing CTE+ tall fescue exhibited increased respiration than those receiving rhizodeposits from E− tall fescue (Van Hecke et al., 2005). In contrast, Franzluebbers et al. (1999) found that tall fescue pastures with high CTE infection frequencies (65-94%) supported less soil microbial biomass and respiration than pastures with low infection (0-29%). Buyer et al. (2011) further observed that mesocosms planted with CTE+ tall fescue exhibited decreased abundance of gram-positive bacteria and arbuscular mycorrhizal lipid biomarkers compared to E− tall fescue, in addition to decreased capacity for soil microbial utilization of several substrate types such as carbohydrates and amino acids. A similar mesocosm experiment by Jenkins et al. (2006) found reduced archaea and gram-positive bacterial communities in bulk soil and reduced delta-proteobacterial communities in the rhizosphere within clay loam soils in CTE+ tall fescue compared to E−, along with endophyte-reduced rhizosphere Planctomycetes communities in loamy sand. Some endophyte-related effects on belowground communities have demonstrated influences on aboveground plant communities. For example, biomass of three tree species, and survival of one species (out of nine studied), were reduced when grown in soil conditioned by long-term establishment of CTE+ tall fescue compared to E− tall fescue due to differences in soil microbial communities (Rudgers and Orr, 2009).

In addition, few studies have investigated the impact of aboveground E. coenophiala symbiosis on additional symbioses within or in close association with tall fescue. This is surprising given that plants associate with a multitude of interacting microbes, including heterotrophic symbionts and endophytes that simultaneously demand
plant resources (Partida-Martinez and Heil, 2011). Some findings suggest that CTE+ tall fescue inhibits belowground symbionts such as AMF within shared host plants and in surrounding soil (Chu-Chou et al., 1992; Guo et al., 1992; Mack and Rudgers, 2008). These effects of CTE+ tall fescue can even extend to inhibition of root AMF colonization in other plants, yet similar effects may not be observed with novel endophytes such as AR542 NTE (Antunes et al., 2008). The effects of *E. coenophiala* symbiosis on other above or belowground symbionts either within or in close proximity to tall fescue, such as dark septate endophytes (DSE) in roots or rhizobia in neighboring legumes, is an area that warrants further study to delineate the current and future ecosystem impacts of this widespread symbiosis, especially with respect to unique host-endophyte genetic combinations.

1.7. A model system: Using the *E. coenophiala*–tall fescue symbiosis to study plant-microbe-soil interactions

Evidence described in the preceding sections suggests that manipulating constitutive symbioses between asexual *Epichloë* endophytes and cool-season grasses could provide a model system in which to explore the complex role of aboveground plant-microbe interactions on belowground ecosystems and soil properties. These symbioses are increasingly prevalent across global ecosystems, especially in agronomic grasslands such as pastures that support animal production. They are relatively easily manipulated and characterized, both in terms of fungal presence and host-symbiont genetic identities, and vertical transmission of *E. coenophiala* to grass progeny ensures that, once inserted (and persistence and transmission verified) endophyte treatments should remain intact for long term ecosystem assessment.
Researchers have often studied the effects of the most common endophyte symbiosis (CTE) within tall fescue on ecosystem characteristics such as plant and herbivore communities. Fewer have investigated the effects of this symbiosis on soil properties and processes, or on other microbial symbioses potentially maintained by the host plant in addition to \textit{E. coenophiala}. In addition, researchers are just beginning to assess how genetic manipulation of endophyte-grass combinations, such as the increased development and utilization of NTE strains within select tall fescue cultivars, will modify these interactions. Therefore, in this dissertation, I explored the following questions: 1) How does manipulating \textit{E. coenophiala} presence and strain in tall fescue affect microbial symbioses in neighboring plants, such as N$_2$ fixation by rhizobia in legumes; 2) How do these manipulations of \textit{E. coenophiala} affect other, simultaneous, microbial symbioses within tall fescue, such as belowground fungal colonization; 3) How does manipulating \textit{E. coenophiala} presence and tall fescue–endophyte genotype impact concomitant belowground fungal symbioses and plant–microbe response to predicted climate change factors such as warming and increased precipitation?
1.8. Figures

**Figure 1.1** Classifications of interactions between species, such as between a plant and a microbe, between two plants, or between two microbes, according to the net effect of interaction. In Bronstein (1994).

**Figure 1.2** Hierarchy of biotic and abiotic factors influencing mycorrhizal interactions. In Johnson et al. (1997).
Figure 1.3 Conditions of relative N and P availability predict host-AMF interactions. In Johnson (2010).
Chapter Two

*Epichloë coenophiala* symbiosis alters nitrogen source of tall fescue host, but not nitrogen fixation in co-occurring red clover¹

2.1. Introduction

Tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort = *Lolium arundinaceum* (Schreb.) Darbysh. = *Festuca arundinacea* Schreb.) is a widely used cool-season forage grass in the Southeast United States. It covers over 14 million hectares of pasture area in this region, a large proportion of which hosts an aboveground asexual fungal endophyte *Epichloë coenophiala* (Morgan-Jones & W. Gams) [= *Neotyphodium coenophialum* (Morgan-Jones & W. Gams) = *Acremonium coenophialum* Morgan-Jones & W. Gams] (Shelby and Dalrymple, 1987). The symbiotic relationship with *E. coenophiala* can increase tall fescue’s drought tolerance (Arachevaleta et al., 1989; Bouton et al., 1993; Elmi and West, 1995), insect and nematode resistance (Clay et al., 1993; Kimmons et al., 1990), and competitive ability in mixed species communities (Hill et al., 1991) relative to uninfected tall fescue, and is thus often considered a defensive mutualism (Clay, 1988). However, one of the defensive mechanisms provided to tall fescue by common toxic endophyte strains of *E. coenophiala* is ergot alkaloid production. The deleterious effects of these compounds on animal performance and health, such as reduced heat tolerance, weight gain, and reproductive success, have been reviewed in detail (Schmidt and Osborn, 1993; Strickland et al., 2011; Strickland et al., 1993). To retain many beneficial characteristics of the grass-endophyte symbiosis while reducing toxicity to livestock, multiple strains of the endophyte, which do not produce ergot

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alkaloids, have been isolated from wild populations for selection and use in tall fescue-based pastures (Bouton et al., 2002). Whereas common toxic endophyte effects on plant communities have been heavily studied [e.g., (Rudgers and Clay, 2007)], only recently have the effects of so-called non-toxic, novel endophytes on plant and soil communities and ecosystem dynamics been examined [e.g., (Rudgers et al., 2010; Thom et al., 2014; Yurkonis et al., 2014)].

Common toxic endophyte-symbiotic (CTE+) tall fescue has often demonstrated enhanced competitive ability relative to other plant species with time [e.g., (Clay et al., 2010)], reducing plant diversity in mixed species stands (Iqbal et al., 2013; Rudgers et al., 2010) compared to uninfected (E−) tall fescue. This could be a particular challenge for utilizing legumes, which are added to pastures to provide increased forage quality and added N fertility via biological N fixation (BNF) in root nodules with diazotrophic bacterial symbionts such as Rhizobium spp. [e.g., (Sleugh et al., 2000), see (Nelson and Moser, 1994)]. Few studies have examined the specific effect of CTE+ tall fescue on clover when grown together, though a recent greenhouse study found no effect (Dirihan et al., 2015). In contrast, three genotypic strains of Epichloë festucae var. lolii (Latch, M.J. Chr. & Samuels) [= Neotyphodium lolii (Latch, M.J. Chr. & Samuels) = Acremonium lolii Latch, M.J. Chr. & Samuels], another asexual fungal endophyte species infecting perennial ryegrass, decreased white clover growth in mixture, but differences between endophyte strains were not attributed to strain-specific alkaloid profiles (Sutherland et al., 1999). Root and leaf extracts of red fescue infected with Epichloë festucae reduced seed germination of red and white clover (Vázquez-de-Aldana et al., 2011), and E. festucae var. festucae -infected red fescue can inhibit red clover biomass
production and reduce growth of other legumes when grown in mixture (Vázquez-de-Aldana et al., 2013). Furthermore, in tall fescue, Peters and Mohammed Zam (1981) found reduced germination and root growth of red clover and birdsfoot trefoil (*Lotus corniculatus* L.) when subjected to tall fescue extracts of unknown endophyte status, and Springer (1996) later found that extracts from E− and CTE+ tall fescue reduced red clover germination and root growth.

Inhibition of forage legumes grown in mixture with CTE+ fescue may be due to allelopathic effects (Springer, 1996; Sutherland et al., 1999; Vázquez-de-Aldana et al., 2013; Vázquez-de-Aldana et al., 2011), or to other competitive effects such as increased soil moisture stress or decreased light interception (Staley and Belesky, 2004). Yet, because formation of bacterial symbiosis for BNF and fixation activity is linked to legume growth and development [e.g., (Delves et al., 1986; Robson et al., 1981)], we must consider whether endophyte-infected tall fescue influences those characteristics that may contribute to inhibition of legumes.

Alterations in nutrient dynamics in neighboring tall fescue plants and the surrounding soil may influence legume growth and N fixation activity. CTE+ tall fescue can accumulate more nutrients such as P, Ca, Zn, and Cu in root tissue than uninfected plants (Malinowski et al., 2000), though specific nutrient uptake dynamics vary widely according to both host and endophyte genotype, especially in response to nutrient limitation (Malinowski and Belesky, 1999). Increased N use efficiency and activity of N assimilation enzymes in CTE+ tall fescue (Arachevaleta et al., 1989; Lyons et al., 1990) may also alter long-term N pools in mixed species stands.
Fungal endophyte symbiosis with tall fescue can also impact soil microorganisms and alter C and N cycles (pools and trace gas flux) (Buyer et al., 2011; Franzluebbers et al., 1999; Iqbal et al., 2013; Rojas et al., 2016). Stands with higher endophyte-infection frequencies contain more soil C and N than E− stands or stands with low frequencies of infected tall fescue, presumably due to decreased microbial activity or altered plant inputs (Franzluebbers et al., 1999; Guo et al., 2015; Iqbal et al., 2012). Therefore, because factors such as nutrient availability can influence non-symbiotic N fixation in grassland soils (Zechmeister-Boltenstern and Kinzel, 1990), non-symbiotic N fixing soil microorganisms may also be affected by CTE+ tall fescue, which has further implications for altered N-pools and dynamics in pastures.

Characteristics of N cycling in terrestrial systems can be assessed by measuring the ratio of naturally occurring $^{15}$N and $^{14}$N stable isotopes in plant or soil material and expressing the results as $\delta^{15}$N, or deviation in the ratio of $^{15}$N: $^{14}$N natural abundance measured in each sample from the standard ratio of 0.0036765 measured in atmospheric N$_2$ and calculated in parts per thousand, also called per mil (‰) (Junk and Svec, 1958; Mariotti, 1983). One key assumption with this approach is that rapid biological transformations of N discriminate against the heavy $^{15}$N form, resulting in products that are $^{15}$N-depleted relative to the lighter $^{14}$N isotope, and these products may be leached, volatilized, or taken up by plants (Pörtl et al., 2007; Templer et al., 2007). Substances enriched in $^{15}$N thus generally accumulate in soil with time, and include highly stable soil organic matter (Shearer et al., 1974). The $\delta^{15}$N of plant or soil material may be interpreted as reflecting the integrated $\delta^{15}$N of its N source, in addition to isotopic fractionation, gains, losses, or mixing of N pools within the plant (Evans, 2001;
Robinson, 2001). For example, some studies in grasslands have utilized the $^{15}$N natural abundance method, in which depleted foliar $^{15}$N in plant species growing in mixed stands with clover demonstrate transfer of $^{15}$N-depleted clover-fixed N to non-legumes (Gubsch et al., 2011; Temperton et al., 2007). The same method has been utilized to examine transfer between N-fixing and non-N-fixing trees (Hoogmoed et al., 2014). Legumes rely heavily on atmospheric $\text{N}_2$, which is fixed through bacterial symbiosis and undergoes further slight fractionation toward the lighter $^{14}$N form (Delwiche and Steyn, 1970); thus, legumes naturally exhibit more depleted $\delta^{15}$N than non-fixing plants in most ecosystems (Virginia and Delwiche, 1982).

Little work has yet investigated the effects of novel endophyte strains on the plant and soil biological processes described above, but some studies suggest that cultivar and endophyte type influence community-scale effects of the symbioses. Whereas stands of novel endophyte-symbiotic (NTE+) or E− tall fescue are more beneficial for animal performance, having reduced toxicity (Bouton et al., 2002), they are not necessarily as persistent as CTE+ tall fescue (Hopkins and Alison, 2006). NTE+ tall fescue may impact plant species abundance and invertebrate community structure differently than CTE+, but specific effects also differ between tall fescue cultivars (Rudgers et al., 2010; Yurkonis et al., 2014). In addition, because some consequences of endophyte infection, such as increased drought resistance (Elmi and West, 1995), the inhibition of legume seed germination (Peters and Mohammed Zam, 1981; Springer, 1996), or effects on soil microbial community composition (Rojas et al., 2016), are not specifically linked to alkaloid production (which is a primary difference between the novel and common toxic
strains) the question remains whether novel endophytes elicit similar effects on both symbiotic and non-symbiotic BNF.

To examine the effects of CTE+ and NTE+ tall fescue on symbiotic and non-symbiotic BNF and concomitant N-usage in tall fescue, I measured the natural abundance of $^{15}$N stable isotope ratios in plant and soil samples in addition to estimating potential N$_2$-fixation activity in free-living, non-symbiotic soil bacteria using the acetylene reduction assay. I hypothesized that in mixed species plots: 1) tall fescue infected with CTE and NE strains will competitively utilize more N and differentially interact with red clover and soil microbial communities compared to endophyte-free tall fescue, reducing symbiotic and non-symbiotic BNF through decreased abundance and growth of neighboring red clover and altered soil microbial communities; 2) differential effects on BNF between endophyte strains will elicit long-term changes in size and isotopic signature of soil N pools.

2.2. Materials and Methods

2.2.1. Site Description and Study Design

This study was conducted at the University of Kentucky Spindletop Research Farm in Lexington, Kentucky (38°6'29"N, 84°29'31"W). The location receives an average annual precipitation of 1163 mm, and has an average annual summer temperature of 23.8 °C and a mean annual winter temperature of 1.6 °C (Ferreira et al., 2010). The soil type was a well-drained Bluegrass-Maury silt loam, which is a fine, mixed, semi-active, mesic Typic Paleudalf that weathered from a silty loess mantle over clayey phosphatic limestone residuum (Soil Survey Staff et al., 2014). Prior to site preparation, this location was an established hayfield containing predominantly tall fescue (‘Select’
variety, endophyte-free), and <5% each of Kentucky bluegrass (*Poa pratensis* L.), nimblewill (*Muhlenbergia schreberi* J.F. Gmel.), and alfalfa (*Medicago sativa* L.) (Flynn et al., 2008). After site clearing and before plot establishment, seven T0 soil samples from 5.0 cm diameter soil cores collected across the study area to a depth of 10 cm were characterized as having 5.81 pH, 2.25% C, 0.25 total %N, and 184 mg P kg⁻¹ soil (Iqbal et al., 2013).

A randomized complete block design (RCBD) containing 30, 2 x 2 m square plots divided among six blocks with five plots each was established on April 10, 2008. Each of the five plots within the six blocks were broadcast with 11.2 kg/ha tall fescue (*Schedonorus arundinaceus* Schreb) seeds in monoculture containing one of the following five fungal endophyte treatments: endophyte-free (E⁻), infected with the common toxic endophyte *E. coenophiala* (CTE+), infected with one of two novel non-toxic endophyte strains (AR542 NTE+ or AR584 NTE+; AR = AgResearch, Hamilton, New Zealand), or a seed mixture containing 25% each of the four previous treatments (EMix). Tall fescue seeds planted in this experiment were from a pasture demonstration farm (PDF) variety provided by the Samuel Roberts Noble Foundation, which recently registered the PDF-AR584 endophyte combination as ‘Texoma’ MaxQ II tall fescue (Hopkins et al., 2011). Individual plots in this study were spatially separated by 1 m alleyways sown with Kentucky bluegrass (*Poa pratensis* L.). All aboveground vegetation in the plots was mowed to a height of 10 cm once per year during the winter (December—February) after plot establishment in 2008. Collection of aboveground plant biomass to a height of 10 cm within a randomly placed 50 x 20 cm quadrat in each plot occurred in September 2011. Endophyte treatments were checked in May 2010, with 20
individual tillers harvested per plot and assayed for endophyte presence using an immunoblot assay and for alkaloid potential using genetic screening (Takah and Young, 2014). At that time, endophyte infection frequencies of the plots were as follows: E− 0.83% infected, CTE+ 84.2% infected, AR 542 NTE+ 83.7% infected, AR584 NTE+ 96.9% infected, and EMix 75.9% infected overall, with 49% NTE+ and 27.5% CTE+.

2.2.2. Sample Collection and Handling

Plant Composition and Forage Types

Whereas only tall fescue was planted at establishment in 2008 and remained the dominant species in each plot, plant community composition across plots had diverged, especially in E− plots, to include an abundance of other graminoid and forb species by 2010 (Iqbal et al., 2013) and included up to 20 species by the time of plant sampling for this study in 2011 (McCulley et al., unpublished data, 2011). Plant species commonly found in the plots included Kentucky bluegrass (found in 100% of plots with an average of 4.8% relative abundance), crabgrass (*Digitaria* spp.; 97%, 8.5%), marestail (*Conyza canadensis* L.; 93%, 8.5%), and nimblewill (87%, 3.7%). These species are presumed to have either germinated from the seedbank or arrived through various natural mechanisms of plant succession such as wind-blown seeds or other vectors. One species present at the time of this study in each of the plots was red clover (*Trifolium pratense* L.), a cool-season perennial legume which has agronomic value for use in mixed species pastures for forage and animal production (Taylor, 2008). Despite the presence of other legumes in this location in previous years, such as alsike clover (*Trifolium hybridum* L.) in treatment plots in 2010 (Iqbal et al., 2013), and alfalfa (*Medicago sativa* L.) presence prior to study
establishment (Flynn et al., 2008), no legumes other than red clover were detected in our study plots in fall 2011.

In September 2011, one sample each of the following three forage types was collected from within the 30 study plots, yielding a total of 90 forage samples: a red clover plant (RC), a tall fescue plant growing in close association with the collected red clover [maximum 8 cm distance between plants; TF(+RC)], and a tall fescue plant spatially isolated from red clover within the plot [minimum 45 cm distance; TF(−RC)]. All forage samples were oven-dried at 55 °C for 48 hr and ball-ground for storage until analysis. At the time of plant harvest in September 2011, the relative percentage abundance of every plant species present in each study plot was visually estimated (to 0.1% cover) using the vegetative canopy coverage scale of Daubenmire (1959).

**Soil Samples with Time**

Two or three composited 1.5 cm diameter soil cores taken to a depth of 10 cm from each of the 30 plots were sampled periodically after site establishment T₀ sampling in 2008. Available soil samples collected for previous research which were used to assess long-term soil N pools from each treatment in this study were: seven ball-ground, dried T₀ soils from pre-establishment in April 2008, March 2010 soils from each plot (n=30) , and May 2011 soils (n=30) that were sieved to 2 mm and stored fresh at −80 °C. Soils were also collected from each plot (n = 30) during October 2012 and 2013 by compositing three 1.5 cm soil cores per plot taken to a depth of 10 cm, and sieved to 2 mm and stored fresh at −80 °C (2012) or −20 °C (2013). I therefore utilized a total of 127 soil samples throughout this study.
2.2.3. Stable Isotope Analysis in Forage and Soil Samples

Before measuring the natural abundance of $^{15}$N, dried and ball-ground forage material was stored in glass vials. Field-fresh soil subsamples from each study year, which were previously sieved and frozen for storage, were dried at 105 °C for 48 hr, ball-ground, and then further dried at 55 °C overnight immediately before $^{15}$N analysis. Based on preliminary tests for appropriate sample weights to avoid measurement errors and maximize precision, 5 mg of forage or 30 mg of soil material was weighed into pre-cleaned tin cups and combusted on a Costech Elemental Analyzer (ECS 4010) attached to a Finnigan Delta$^{Plus}$XP continuous flow isotope ratio mass spectrometer (CF-IRMS). CF-IRMS analysis provided measurements of total N concentration (%) and $^{15}$N: $^{14}$N isotopic ratio for each sample. Then, for each sample, $\delta^{15}$N was calculated as: $\delta^{15}$N (‰) = ((R$_{sample}$ / R$_{standard}$) − 1) x 1000), in which R$_{sample}$ and R$_{standard}$ are the $^{15}$N: $^{14}$N ratios measured in each sample and in atmospheric N$_2$, respectively. Repeated measurements of in-house and international standards were included throughout each run sequence (n = 4) in order to calibrate sample values against known ‰ values of $\delta^{15}$N. Isotope measurements were generally reproducible within ± 0.2‰ (standard error) for $\delta^{15}$N values.

2.2.4. Acetylene Reduction Assays (ARA) in Soil Samples

To evaluate the potential activity of free-living N fixing microorganisms in soil samples, laboratory incubation assays of acetylene reduction to ethylene, where acetylene is provided as an alternative substrate for the nitrogenase enzyme responsible for biological N$_2$-fixation activity, were performed using a method adapted from Hardy et al. (1968) and Döbereiner et al. (1972). Because most soil samples available for this study were previously sieved and fresh-frozen (e.g., May 2011 soils), this study utilized sieved
bulk soil samples rather than soil cores assayed in situ, as are often done in field studies of nitrogen dynamics [e.g., (Keuter et al., 2014; Strauss et al., 2012)]. In addition, because free-living biological N fixation by soil microorganisms varies seasonally (Belnap, 2002; Watanabe et al., 1978), only soils from October 2012 and 2013 were compared for changes in activity with time, whereas soils from May 2011 were used only to detect differences resulting from endophyte treatments. Six grams dry weight equivalent (DWE) each of thawed, field-moist soil samples from May 2011, October 2012, and October 2013 were weighed into 50 mL plastic centrifuge tubes with O-rings and septum installed in the caps, adjusted to 30% gravimetric soil moisture content, and allowed to pre-incubate at 20 °C, uncapped and covered with Parafilm, for 2 d to equilibrate from storage conditions. To avoid any physiological effects of long-term acetylene exposure on microorganisms (David and Fay, 1977) or possible long-term selection for acetylene use within the soil microbial community, which might interfere with treatment effects, I chose an assay incubation time of 6 hr.

Acetylene (C2H2) gas was generated by adding distilled H2O to evacuated calcium carbide granules (Fisher Scientific, #C57-500) in a glass serum bottle. For each soil sample, C2H2 was injected into assay tubes to 0.1 atm. Blank tubes, containing no soil but receiving C2H2, were included during each assay to correct for ethylene (C2H4) impurities in laboratory-generated C2H2 gas. Assay tubes were incubated at 20 °C in the dark for 6 hr after injection. Gas sub-samples were withdrawn from each tube at 6 h and placed in pre-evacuated 13 mm crimp-top glass vials, then stored under water to prevent leakage until gas chromatography (GC) analysis within 24 hr. The C2H4 concentration in 100 µL injections of each stored sample was measured on a Shimadzu GC-14A (Shimadzu
Scientific, Columbia, Maryland, USA) equipped with a Poropak R column (80—100 mesh, 2m x 2mm). Samples were passed through a flame ionization detector (FID) using an injection temperature of 70 °C, an initial column temperature of 50 °C, and a final detector temperature of 155 °C, and using N₂ as a carrier gas at 200 kPa. After calculating injected sample concentrations using pure C₂H₄ gas (100 ppm C₂H₄ in He, Matheson Tri-Gas Inc., #GMT10325TK, Twinsburg, OH) as a standard and subsequently adjusting for C₂H₄ impurity from blank assay tubes, the amount of C₂H₄ evolved from C₂H₂ during the 6 h incubation assay per gram DWE soil was calculated for each sample as nmol C₂H₄ g⁻¹ dry soil.

2.2.5. Statistical Analysis

I tested for statistically significant effects (α = 0.05) of endophyte treatment, forage type, and year of soil sampling, where applicable, on measured plant and soil parameters using the PROC MIXED procedure in SAS (9.3 SAS Institute Inc., Cary, NC, USA). To examine differences in δ¹⁵N between forage types from each plot, the data were analyzed as a split-plot design within the experimental randomized complete block design (RCBD), with endophyte treatment and forage type as fixed effects, and both block and the interactive effects of treatment and block specified as random effects. Significant endophyte treatment effects and changes with time were analyzed for δ¹⁵N in soil samples using the previously described mixed modeling procedure in SAS, though with no split-plot designation. Endophyte treatment and year of sampling were modeled as fixed effects, with block specified as a random effect, and a repeated measures statement for each block x treatment by year was added to detect significant changes with time. Results from soil ARAs were statistically analyzed two ways, in which: 1) the fixed
effects of endophyte treatment and sampling year (October 2012 and 2013) were examined using repeated measures as described above for analysis of soil δ¹⁵N; 2) 2011 soils were individually analyzed for only the fixed effects of endophyte treatment without the repeated measures statement. Individual relative abundance estimates of tall fescue and red clover in 2011 were also analyzed for fixed effects of endophyte treatment and random effects of block using PROC MIXED. When significant main or interactive effects were found, significant differences between individual treatments, years, or forage types for all analyses were determined by comparing the LSMEANS using the PDIF option in SAS.

2.3. Results

2.3.1. Plant Composition

Endophyte infection treatments resulted in significant differences in the relative abundance of tall fescue (Fig. 2.1A; p = 0.0021; F₄, ₂₀ = 6.17), in which CTE+ plots contained approximately 42% more tall fescue cover than E− plots and approximately 32% more than in AR542 NTE+ plots. The relative abundance of red clover cover was not significantly affected by endophyte treatments (Fig. 2.1B; p = 0.1241; F₄, ₂₀ = 2.06) and averaged 15% (± 1.86 S.E.) across plots, although red clover tended to exhibit greater abundance in plots with significantly reduced abundance of tall fescue, such as E− and AR542 NTE+ plots. Endophyte infection significantly reduced the abundance of graminoid species other than tall fescue (Fig. 2.1C; p = 0.0379; F₄, ₂₀ = 3.12), with CTE+ and both NTE+ treatments containing approximately 12% less other graminoid cover, on average, than E− plots. The relative cover of forb species, excluding red clover, was also significantly affected by endophyte infection (Fig. 2.1D; p = 0.0021; F₄, ₂₀ = 6.18), in that
CTE+ plots contained approximately 20% less forb cover than E− plots, and 7.5% less forb cover than EMix plots.

2.3.2. Stable Isotope Analysis in Plant and Soil Samples

Forage Types

While endophyte infection status did not significantly alter the natural abundance of $^{15}$N ($\delta^{15}$N) within associated red clover (RC) samples ($p > 0.05$), $\delta^{15}$N in tall fescue samples differed significantly within both TF(+RC) and TF(−RC) forage type and endophyte treatment (Fig. 2.2A, Endophyte x Forage $p = 0.016$; $F_{8, 45} = 2.71$). As expected, $\delta^{15}$N of RC samples were significantly more depleted than all tall fescue samples (Fig. 2.2A; all $p < 0.05$), indicating RC utilization of primarily $^{15}$N-depleted N products via symbiotic N$_2$-fixation. This forage type effect was consistent across endophyte treatments. For tall fescue growing near red clover, TF(+RC), samples from plots infected with either the common toxic endophyte (CTE+) or the novel endophyte AR584 (AR584 NE+) were significantly more depleted in $\delta^{15}$N compared to AR542 NTE+ plots (Fig. 2.2A), but were not different than E− tall fescue. However, when located away from red clover, TF(−RC), samples from only endophyte free (E−) plots were significantly depleted compared to all other endophyte treatments ($p < 0.05$).

The N concentration (%) of RC samples differed significantly as a result of endophyte treatment, with RC from E−, AR542 NE+, and EMix plots containing significantly higher N than from CTE+ plots (Fig. 2.2B, Endophyte x Forage $p = 0.0446$; $F_{8, 45} = 2.21$). Within tall fescue samples, no significant differences in N were measured between endophyte treatments, and this effect was consistent regardless of whether tall fescue was located near red clover or not.
Soil Samples

Averaged across endophyte treatments, soil $\delta^{15}$N steadily and significantly declined during each year of analysis (Fig. 2.3A, Year $p < 0.0001$; $F_{3, 92} = 41.62$), whereas no significant differences were measured between endophyte treatments either individually (Endophyte $p = 0.8785$; $F_{4, 92} = 0.30$) or over time (Endophyte x Year $p = 0.1303$; $F_{12, 92} = 1.52$).

The N concentration measured in soil samples was significantly affected by the interaction between endophyte treatment and year of analysis (Fig. 2.3B, Endophyte x Year $p = 0.0425$; $F_{12, 92} = 1.91$), but increases over time were small (on average, +0.0263 % N between 2010 and 2013). The Endophyte x Year interaction also appeared to be driven by slightly higher N in AR584 NTE+ and AR542 NTE+ plots in most years, with the least N contained in EMix plots (Fig. 2.3B).

2.3.3. Acetylene Reduction in Soil Samples

No significant endophyte effects on potential free-living N fixing activity were detected in either May 2011 (Fig. 2.4A, Endophyte $p = 0.1928$; $F_{4, 10} = 1.87$), or October 2012 and 2013 soils (Fig. 2.4B, Endophyte $p = 0.9176$; $F_{4, 24} = 0.23$). In May 2011 soils, free-living N-fixing organisms showed slightly higher activity in CTE+ and AR584 NTE+ plots, but differences were not significant. Overall potential activity significantly increased between October 2012 and 2013 when analyzed together in a repeated measures model (Fig. 2.4B, Year $p = 0.0001$; $F_{1, 24} = 21.35$), though no significant endophyte effects or interactive effects of endophyte treatment and year (Endophyte x Year $p = 0.0936$; $F_{4, 24} = 2.25$) were found.
2.4. Discussion

The infecting strain of *E. coenophiala* and the proximity of red clover influenced the proportion of biologically-fixed N\textsubscript{2} utilized by tall fescue, as indicated by $\delta^{15}$N in tall fescue tissue. However, there were no significant effects of either CTE+ or NTE+ tall fescue on $\delta^{15}$N within red clover, $\delta^{15}$N in soil samples, or the potential activity of non-symbiotic N\textsubscript{2}-fixing soil microorganisms. The $\delta^{15}$N in soil samples from each treatment at this site steadily declined over time, while non-symbiotic N\textsubscript{2} fixation activity increased significantly between the last two study years. These results suggest that endophyte infection in tall fescue may not significantly influence symbiotic or non-symbiotic N\textsubscript{2}-fixation capacity in mixed species pastures, but different endophyte strains can affect the ability of tall fescue to utilize fixed-N\textsubscript{2} produced by neighboring red clover or free-living soil microorganisms.

My first hypotheses, in which I expected CTE and NE infection to increase uptake of biologically fixed N in tall fescue and alter biological N cycling both in neighboring red clover and in free-living soil microorganisms, were unsupported by the results. Uptake of $^{15}$N-depleted N in tall fescue grown near red clover was not altered solely by endophyte infection, although endophyte strain did appear to influence uptake of $^{15}$N-depleted N in tall fescue (Fig. 2.2A). When grown near red clover, tall fescue infected with the novel AR542 endophyte accessed significantly less $^{15}$N-depleted N than either CTE+ or AR584 NTE+ tall fescue. This suggests that although neither endophyte infection nor toxicity of endophyte strain in tall fescue alters access to immediately proximate products of biological N\textsubscript{2} fixation compared to E− plots, plants with different endophyte strains differ in their ability to gain fixed N from neighboring red clover. This
...dynamic was not observed in tall fescue samples collected within the same endophyte treatment plots yet spatially isolated from red clover. In TF(–RC) samples, only E– tall fescue exhibited significant δ¹⁵N depletion compared to either CTE+ or NTE+ tall fescue. The N isotope signature of tall fescue may be altered if mycorrhizal networks were impacted by endophyte strain or proximity to red clover, because transfer of N through mycorrhizal networks is known to fractionate against ¹⁵N (Hobbie and Ouimette, 2009). For example, greater transfer of biologically-fixed N from red clover to tall fescue may have occurred through increased mycorrhizal networks (Haystead et al., 1988; Mårtensson et al., 1998) in TF(+RC) samples compared to TF(–RC) samples or in E– plots compared to endophyte-infected plots (Chu-Chou et al., 1992; Guo et al., 1992). I found no evidence of endophyte-associated significant differences in N dynamics from analysis of plant or soil δ¹⁵N or in assays of free-living bacteria activity. Endophyte presence and strain had no significant effect on δ¹⁵N in red clover grown adjacent to tall fescue in this study (Fig. 2.2A), despite significant changes in tissue N (Fig. 2.2B). My findings support earlier reports suggesting that neither endophyte nor alkaloid presence in tall fescue is the mechanism responsible for reduced legume seedling germination and growth (Dirihan et al., 2015; Springer, 1996; Staley and Belesky, 2004), I had originally hypothesized that differences in plant competition between legumes and tall fescue resulting from endophyte presence or strain would subsequently affect N₂-fixation capacity. However, although the effects of endophyte presence and strain on utilization of ¹⁵N-depleted products, such as biologically-fixed N₂, in red clover were not significant in this study, the significant differences measured in N concentration of red clover (Fig. 2.2B) reflect the trends observed in δ¹⁵N (Fig. 2.2A). N concentration of red
clover tissue was significantly lower in plots containing CTE+ or AR584 NTE+ tall fescue compared to AR542 NTE+ plots, and somewhat lower than E− or EMix plots. In a reversal of this trend, δ15N of red clover was most heavily depleted in CTE+ or AR584 NTE+ plots, though not significantly. This suggests that although red clover in CTE+ or AR584 NTE+ plots relied most heavily on biological N fixation, less N was incorporated into aboveground tissue. Schipanski and Drinkwater (2012) estimated that in red clover-orchardgrass mixtures, N fixation activity increased by 15% due to transfer of fixed N between species. García Parisi et al. (2014) also found that asexual Epichloë spp. infection of annual ryegrass almost doubled N fixation activity and biomass in neighboring white clover despite a reduction in nodulation. These could explain my results, in which products of higher N2-fixation in red clover may have been increasingly transferred to other plant species such as tall fescue in the TF(+RC) CTE+ and AR584 NTE+ treatments, especially compared to AR542 NE+, while competitive ability of E− tall fescue for N seemed little impacted by proximity to red clover. Although this endophyte-specific mediated increase in tall fescue’s competitive ability is supported by significantly increased CTE+ tall fescue cover compared to AR542 NE+, coupled with trends for decreased red clover cover in CTE+ plots compared to AR542 NTE+ (Fig. 2.1), no such biomass trends were observed for AR584 NTE+ plots, suggesting other competitive mechanisms were influenced by this strain of the endophyte. I suggest that differential mechanisms and effects of endophyte strain will impact nutrient transfer dynamics and legume N2-fixation, as well as legume and forage nutritive value.

Few studies have investigated the effects of endophyte-infected tall fescue on soil microbial communities, and, to my knowledge, no studies have examined these effects on
biological N$_2$-fixing activity by free-living soil diazotrophs. Iqbal et al. (2012) found higher total microbial biomass in CTE+ plots compared to E−, whereas Franzluebbers et al. (1999) measured lower microbial biomass and respiration in soils associated with tall fescue with a high endophyte infection frequency compared to low endophyte infection frequency. I was therefore surprised to find no significant effects of endophyte infection on assays of acetylene reduction in soils from each treatment over multiple years. Though unmeasured in this study, I expected potential endophyte-associated differences in soil microbial biomass, as measured in other studies, to elicit differences in activity of non-symbiotic N$_2$-fixing soil microorganisms. However, grasslands are known for having lower global rates of non-symbiotic N fixation relative to other ecosystems such as tropical rainforests (Cleveland et al., 1999), and thus the proportion of N$_2$-fixing microbes within the microbial biomass may have been too small at our site to be affected by potential changes in total microbial biomass. The low overall activity at this site may have also resulted from incubating samples in the dark, which excluded autotrophic diazotrophs such as cyanobacteria, or from lack of glucose amendment prior to incubation to decrease carbon limitation and increase activities. In May 2011 soils (Fig. 2.4A), non-significant trends in my results showed increased non-symbiotic N$_2$-fixation activity in CTE+ soils compared to E− soils. This result complements a study by Franzluebbers and Hill (2005), who found increased microbial biomass N, but reduced microbial biomass C, in soils exposed to E+ tall fescue litter relative to E− tissue, although I caution that I did not consistently observe this effect across years (Fig. 2.4B). It is of interest to note that trends observed in non-symbiotic N$_2$-fixation from May 2011 soils closely followed those observed in δ$^{15}$N of red clover tissue, which indicated a
greater degree of reliance on N$_2$ fixation due to greater demand by CTE+ tall fescue. Non-symbiotic N$_2$-fixation activity in May 2011 was higher in CTE+ and AR584 NTE+ soils than in other treatments, potentially providing further support, although not significant, for the discussion of stimulated biological N$_2$ fixation resulting from increased competition for N and N transfer between grasses and legumes in mixed stands. These trends were not observed in soils from October of 2012 or 2013, so it is also possible that some $^{15}$N-depleted N utilized by red clover was a product of non-symbiotic soil microorganisms rather than symbiotic BNF.

My second hypotheses, which predicted that differences in biological N fixation and N uptake as a result of endophyte presence or strain infecting tall fescue would produce long-term effects on soil N pools, were also unsupported. No consistent differences between endophyte treatments were observed either in soil $\delta^{15}$N (Fig. 2.3A) or potential non-symbiotic N$_2$ fixation (Fig. 2.4B) with time. Lack of endophyte effects on long-term soil N pools was likely because, while I did observe differences in pool access in tall fescue (Fig. 2.2A), no changes were observed in tall fescue tissue N (Fig. 2.2B), which was the dominant plant species in each treatment (Fig. 2.1). Although one may expect to see long-term changes in $\delta^{15}$N resulting from differences observed both in $\delta^{15}$N and N concentration in red clover, in 2011, these changes may have been too small to detect because of a relatively low abundance of red clover in subsequent years (data not shown), and the absence of endophyte treatment effects on the relative abundance (and N fixation) of red clover in 2011 (Fig. 2.1B). Differences in soil $\delta^{15}$N between endophyte treatment plots from 2010—2013 were inconsistent and not statistically significant, although interannual dynamics appeared to pair E− with CTE+ plots, and
AR542 NTE+ with EMix plots (Fig. 2.3A). Statistically significant differences in soil N concentration did occur between endophyte treatments with time (Fig. 2.3B), but these changes in N were very small and did not reflect the treatment patterns observed in $\delta^{15}$N. The relative subtlety of endophyte effects on soil $\delta^{15}$N in this study could potentially be due to relationships between BNF and soil phosphorus. Although much of the relationship between legume N and P requirements across ecosystem characteristics and plant species remains unclear, studies have often shown that adequate P levels are an important control of BNF (Vitousek et al., 2002). Because I assumed BNF to be the primary source of $^{15}$N-depleted N, and low N:P ratios increase N$_2$-fixation (Eisele et al., 1989; Vitousek and Field, 1999), it is possible that differences in N$_2$-fixation between treatments in this study were minimized by naturally high levels of P in these soils from phosphatic limestone parent material (Karathanasis, 1991).

Although no significant endophyte effects on BNF were measured in this study, I found significant changes over time for both $\delta^{15}$N and the activity of free-living N fixing soil microorganisms. This site exhibited significant declines in soil $\delta^{15}$N between 2010 and 2013, which may suggest that either $^{15}$N is being lost or $^{14}$N is accumulating. Many soils exhibit $^{15}$N enrichment over time, because $^{15}$N-depleted forms of N produced through biologically mediated transformations are fractionated against and accumulate in soils as stable organic N while $^{14}$N-enriched inorganic N is lost (Brenner et al., 2001; Menge et al., 2011). However, Brenner et al. (2001) attributed increased $\delta^{15}$N of older soils to eventual P-limitation. This study site’s naturally high soil P levels may have also resulted in relatively less N loss with time compared to other studies. Temperton et al. (2007) further observed that increasing plant species richness in pasture soils decreased
soil $\delta^{15}$N independently of legume effects, and I have also observed increased plant diversity across treatment plots because only tall fescue was planted in 2008 (Iqbal et al., 2013). In addition, decline of $\delta^{15}$N across pasture soil chronosequences was observed by Piccolo et al. (1996), who attributed decreased $\delta^{15}$N to increased inputs of BNF with time. I glimpsed a similar effect in my study through the significant increase in nonsymbiotic BNF between 2012 and 2013 (Fig. 2.4B). Thus, adequate soil P and potentially increased BNF inputs with time may have contributed to steadily decreasing soil $\delta^{15}$N at this temperate grassland site regardless of endophyte treatments.

2.5. Conclusions

This study suggests that regardless of alkaloid profile or toxicity, specific endophyte strain-tall fescue combinations differentially impact the amount of biologically-fixed N$_2$ utilized by tall fescue, though not resultant tissue N, when grown in close association with red clover in mixed species pastures. When spatially distant from red clover, only E$^{-}$ tall fescue could utilize more biologically fixed N. Assays of nonsymbiotic soil microbial N$_2$-fixation in bulk soils did not reveal any endophyte treatment effects, and there were no differences between treatments in soil $\delta^{15}$N over time. A steady decline in average soil $\delta^{15}$N with time at this site might be attributable either to successive closure to N-loss with time, increased biological N fixation inputs, or to minimized P limitations due to phosphatic parent material and adequate rainfall. Different effects of endophyte strain on tall fescue competitive ability and utilization of N produced by N-fixing symbioses are likely to impact nutrient cycling of pastures and therefore should be considered in the development and adoption of new grass-endophyte combinations.
2.6. Figures

**Figure 2.1** Estimates of relative cover (%) in each treatment plot for A) tall fescue, B) red clover, C) other graminoid species, excluding tall fescue, and D) forb species, excluding red clover in September 2011. Within each panel, a, b, c denote significant differences between endophyte treatments ($\alpha = 0.05$), while bars indicate $\pm 1$ S.E. of each average.
Figure 2.2 A) $^{15}$N natural abundance ($\delta^{15}$N) and B) total aboveground plant tissue nitrogen concentration (%) measured in red clover (RC), tall fescue associated with red clover (TF+RC), and tall fescue not associated with red clover (TF-RC) harvested from each endophyte treatment plot in September 2011. Within each forage type, a, b, c indicate significant differences between endophyte treatments ($\alpha < 0.05$; NS – not significant). A, B, C, indicate significant differences between forage type across endophyte treatments in the x-axis labels.
Figure 2.3 A) $^{15}$N natural abundance ($\delta^{15}$N) and B) total N concentration measured in bulk soil samples collected from each endophyte treatment plot with time. A, B, C, D indicate significant differences between average soil $\delta^{15}$N across treatments for the main effect of year ($\alpha = 0.05$) in panel A, although data are presented by endophyte treatment to aid interpretation. Data in panel B are arranged to illustrate the significant interactive effect of endophyte treatment and year ($\alpha = 0.05$). Points and bars represent treatment average ± 1 S.E. In both panels, the dashed line represents the average site $\delta^{15}$N or N measured in T0 bulk soil samples collected immediately prior to plot establishment in 2008, which is provided for reference and thus not included in statistical analyses, and the grey shaded area represents ± 1 S.E.
Figure 2.4 Potential non-symbiotic N₂-fixation results determined via assays of C₂H₂ reduction to C₂H₄ in bulk soil samples from A) May 2011 and B) October 2012 and 2013. No significant effects of endophyte treatment were detected in May 2011 soils, which were not compared to October 2012 and 2013 soils because of confounding differences in seasonal variation of microbial activity. A, B denote significant main effects of year between October 2012 and 2013 ($\alpha = 0.05$). Bars in each panel indicate averages ± 1 S.E.
Chapter Three

Different *Epichloë coenophiala* strains similarly affect belowground fungal colonization of tall fescue roots

3.1. Introduction

Plants form and maintain myriad symbioses with microorganisms such as bacteria, viruses, and fungi. These relationships occur above- and belowground, can be host-specific, and function along a continuum of host-symbiont interactions from parasitism to mutualism. One symbiosis of great ecological and economic importance is that between tall fescue (*Schedonorus arundinaceus*), a non-native cool-season forage grass in the U.S., and *Epichloë coenophiala* (= *Neotyphodium coenophialum*), an aboveground Clavicipitaceous fungal endophyte that specifically associates with tall fescue. Tall fescue inhabits 15 million hectares across the U.S. (Rogers and Locke, 2013), a large proportion of which is infected with *E. coenophiala* (Shelby and Dalrymple, 1987).

*Epichloë coenophiala* most commonly functions as a defensive mutualist that grows intercellularly within tall fescue and subsists on apoplastic sugars and amino acids, and in return enhances drought and mineral stress resistance (Arachevaleta et al., 1989; Bouton et al., 1993; Elmi and West, 1995; Malinowski et al., 2000), increases competitive ability (Hill et al., 1991), growth, and reproduction (Gundel et al., 2013), and produces alkaloid compounds that deter both mammal and insect herbivory (Bush et al., 1993; Porter et al., 1981). Through these effects, *E. coenophiala* infection often reduces plant diversity and gradually increases tall fescue abundance in mixed plant communities (Clay and Holah, 1999; Iqbal et al., 2013). Endophyte-infected tall fescue stands also
accumulate more soil organic carbon (SOC) and total nitrogen (TN) with time compared to uninfected (E−) stands (Franzluebbers et al., 1999; Franzluebbers and Stuedemann, 2005; Iqbal et al., 2012).

Unfortunately, ergot alkaloids produced by the *E. coenophiala* strain found most commonly in the U.S. cause grazing livestock to exhibit well-documented toxicity symptoms, such as impaired heat tolerance, reduced body weight gain and reduced reproductive success, which are encompassed in the term “tall fescue toxicosis” (Strickland et al., 2011). However, *E. coenophiala* strains that naturally do not produce the mammal-toxic ergot alkaloids, yet continue to deter insect herbivory through loline and peramine alkaloid production, have been isolated from native populations of tall fescue and selectively introduced into forage cultivars for decreased livestock toxicity (Bouton et al., 2002), often at cost to the benefits to plant fitness and persistence described above (Bouton et al., 1993). These ‘novel’ or ‘non-toxic’ endophytes (NTE) are increasingly deployed in pastures world-wide, yet we do not fully understand the ecological implications of these symbioses on plant communities, soil properties, and concomitant symbionts in tall fescue.

Another plant-microbial symbiosis of ecological and agronomic importance is one that exists between nearly 80% of land plants and vesicular-arbuscular mycorrhizal fungi (AMF) of the phylum *Glomeromycota* (Schübler et al., 2001; Smith and Read, 2008). AMF are common root symbionts of terrestrial plants, including grasses, increasing water and nutrient uptake in exchange for host photosynthate (Augé, 2001; Smith and Read, 2008). AMF are considered nutritional mutualists, with the availability of nutrients, such as P and N, influencing the relative benefit AMF confer to the host plant. For example,
AMF may be a parasitic sink for plant C when environmental N and P are in abundance, but become more commensalistic or mutualistic when P is limited (Johnson, 2010). In addition to effects on plant hosts, AMF soil hyphal networks can lead to improvement of soil physical properties such as aggregate size and stability, and increased C sequestration, particularly in pasture soils (Duchicela et al., 2013; Miller and Jastrow, 1990).

Another group of belowground endophytic fungi that frequently coexist with AMF are dark septate endophytes (DSE) of the phylum Ascomycota. DSE may perform similar or complementary functions to AMF, but researchers are just beginning to investigate these possibilities (Mandyam and Jumpponen, 2005; Mandyam and Jumpponen, 2014). If DSE produce similar effects to AMF, both symbionts may be important in governing plant productivity and soil properties in pasture ecosystems, such as those dominated by tall fescue.

Little is known about how tall fescue aboveground symbiosis with E. coenophiala affects belowground symbioses with AMF and DSE. Studies show that symbiosis with the common toxic endophyte (CTE+) decreases AMF root colonization rate in tall fescue (Chu-Chou et al., 1992; Guo et al., 1992; Mack and Rudgers, 2008). CTE+ tall fescue also lowered the abundance of AMF lipid biomarker 16:1 o5 cis in soils compared to E− tall fescue (Buyer et al., 2011). In addition, decomposing CTE+ tall fescue thatch reduced AMF colonization rates in other plants, whereas E− and a novel (strain AR542) endophyte-infected tall fescue (AR542 NTE+) did not produce the same effect (Antunes et al., 2008). These studies suggest that compounds produced in CTE+ tall fescue, such as ergovaline, have a negative effect on AMF. However, although symbiosis with a
similar asexual endophyte, *E. festucae* var. *lolii*, also reduced AMF infection in *Lolium perenne* L. (Müller, 2003), Liu et al. (2011) observed that competition between the endophyte and AMF was mitigated in a higher sugar producing host cultivar. Symbiosis with *E. occultans* (C.D. Moon, B. Scott & M.J. Chr.) [= *Neotyphodium occultans* C.D. Moon, B. Scott & M.J. Chr.] in *Lolium multiflorum* Lam. also decreased AMF colonization in E+ plants, yet increased AMF colonization in neighboring E− plants when grown together (Omacini et al., 2006). Field studies of other cool-season grasses hosting similar asexual *Epichloë* species have further revealed that endophyte infection may stimulate host AMF colonization (Novas et al., 2005; Novas et al., 2009; Novas et al., 2011) and contribute to added plant growth (Larimer et al., 2012; Novas et al., 2005). We do not fully understand what causes this divergence in responses between different grass host–endophyte–AMF relationships, nor have there been comprehensive examinations of these relationships for tall fescue in field settings or considering different endophyte strains, DSE, or their impacts on related ecosystem parameters.

To address this knowledge gap, with plant and soil samples collected from a five-year old field study, I examined how CTE and NTE strains of *E. coenophiala* in tall fescue affected root mycorrhizal and DSE colonization, associated shoot and root nutrients, lengths of soil extraradical AMF hyphae, water stable soil aggregates, and C and N within aggregates. I hypothesized that: 1) CTE+ plots would have lower root AMF and DSE colonization rates compared to endophyte-free (E−) or novel endophyte-infected (NTE+) tall fescue; 2) CTE+ plots would have lower extraradical AMF hyphae compared to E− or NTE+ plots due to inhibition by decomposing CTE+ litter and
lowered plant diversity; and 3) These effects would lead to greater water stable soil aggregates and C concentration in E− and NTE+ plots than in CTE+.

3.2. Materials and Methods

3.2.1. Site Description and Study Design

The long-term field study was located in Lexington, Kentucky at the University of Kentucky Spindletop Research Farm (38°6’29”N, 84°29’31”W). This area has average summer and winter temperatures of 23.8 °C and 1.6 °C, respectively, and receives an average of 1163 mm annual precipitation (Ferreira et al., 2010). The soil is described as a Bluegrass-Maury silt loam that weathered from a silty loess mantle over clayey phosphatic limestone residuum, and is a well-drained fine, mixed, semi-active, mesic Typic Paleudalf (Soil Survey Staff et al., 2014). Soil C, N, and P levels in the upper 10 cm at study establishment were 2.25% C, 0.25% N, and 184 mg P kg\(^{-1}\) soil (Iqbal et al., 2013). At the time of this study (May 2013), the mean soil test nutrient levels and Sikora II buffer pH for the study site measured by the University of Kentucky Soil Testing Regulatory Services were as follows: 184.21 mg P kg\(^{-1}\) soil, 90.81 mg K kg\(^{-1}\) soil, 1582.98 mg Ca kg\(^{-1}\) soil, 143.90 mg Mg kg\(^{-1}\) soil, 1.88 mg Zn kg\(^{-1}\) soil, and 6.57 pH.

Prior to this study, the site was a hayfield dominated by ‘Select’ variety endophyte-free tall fescue (Flynn et al., 2008). On 10 April 2008, field plots were established in a randomized complete block design (RCBD) with six blocks comprised of four plots each, resulting in a total of 24, 2 x 2 m squares. Tall fescue seed of a pasture demonstration farm (PDF) variety provided by the Samuel Roberts Noble Foundation was hand-broadcast in monoculture at a rate of 11.2 kg ha\(^{-1}\) in each plot and contained one of four treatments: endophyte-free (E−), infected with the common toxic strain of E.
coenophiala (CTE+), infected with one of two novel non-toxic endophyte strains (AR542 NTE+ or AR584 NTE+; AR=AgResearch, Hamilton, New Zealand). Endophyte frequency, via immunoblot assay, and endophyte strain, via genetic screening (Takah and Young, 2014), were verified in 20 tillers from each plot in May 2010. E− plots were 1% infected, CTE+ plots were 84% infected, AR542 NTE+ plots were 84% infected, and AR584 NTE+ plots were 97% infected. Genetic strain tests confirmed that CTE+ and NTE+ treatments were as planned.

3.2.2. Sample Harvest and Preparation

On 30 May 2013, five years after plot establishment, I harvested aboveground ramets (vegetative clones consisting of 2-4 tillers) of tall fescue with intact belowground roots from three individual plants within each of the 24 plots considered in this study, resulting in a total of 72 plant samples. In addition, three 1.5 cm diameter soil cores were collected and composited for each plot, resulting in 24 soil samples, which were sieved to 2 mm and air-dried to await analyses. Roots were separated from the aboveground portions of each ramet, washed, and dried at 55 °C. After analysis of AMF and DSE colonization rates, all roots per plot were cyclone milled for nutrient analyses. Endophyte presence/absence was verified in individual tillers within each ramet using an Epichloë-specific enzyme-linked immunoblot assay (Hiatt et al., 1999). Tillers comprising six ramets tested as E− in CTE+ or NTE+ plots out of the 72 study ramets, and were excluded from the study. All tillers across ramets were then composited, dried, and milled.
3.2.3. Root Mycorrhizal and DSE Colonization

To measure the rate of mycorrhizal and DSE colonization in tall fescue, I stained root sections with trypan blue and counted presence of AMF infection structures via microscopy (McGonigle et al., 1990). Subsections of dried roots were cleared of cellular contents using 10% KOH, acidified in 2% HCl, and stained with 0.05% trypan blue. Roots were de-stained in 1:1 glycerol: deionized (DI) water for at least 2 d, then arranged on 25 mm microscope slides in two columns of 5 roots each and allowed to dry completely before securing cover slips with polyvinyl lactoglycerol (PVLG; INVAM). AMF colonization rate was measured using a line intersect method at 400 x magnification with 30 total intersections modified from McGonigle et al. (1990). Presence of AMF arbuscules, vesicles, or hyphae were counted at each intersection. Only one count was recorded when multiple structures intersected, with priority given to arbuscules > vesicles > hyphae. Presence of melanized, septate DSE hyphae or microsclerotia was tallied in addition to AMF colonization during microscopy. Total AMF and DSE colonization (%) were calculated as the number of presences divided by the 30 possible views and multiplied by 100.

3.2.4. Plant Nutrients

Total N and P concentrations within milled tall fescue tissue, composited across individual ramets per plot, were measured separately for root and shoot material via wet digestion. All plant N was converted to NH$_3$ (Bradstreet, 1965). Colorimetric determination (%) for NH$_3$ was modified from the Berthelot reaction (Chaney and Marbach, 1962). All plant P was reduced to PO$_4^{3-}$ and colorimetrically determined (%) based on Fiske and Subbarow (1925).
3.2.5. Extraradical AMF in Soil

To estimate the length of extraradical AMF hyphae within bulk soil samples from each plot, I extracted hyphae from a 4 g soil subsample by an aqueous extraction and membrane filter technique modified from Jakobsen et al. (1992) and Rillig et al. (1999). Four grams of soil were dispersed in 100 mL of DI water with an added 12 mL of 35 g L\(^{-1}\) (NaPO\(_3\))\(_6\). Solutions were shaken by hand, sonicated, then allowed to settle for up to 1 hr. Solutions were then passed through a 38 µm sieve to retain hyphae, roots, and organic material, which were washed from the sieve into 250 mL flasks with 200 mL of DI water. These were shaken by hand for 5 s and allowed to settle for 1 min. A 4 mL aliquot was taken from each flask and pipetted into an open syringe attached to a 25 mm Millipore filter holder containing 25 mm diameter nitrocellulose membrane filters with 0.45 µm pore size. The solution was stained with approximately 1 mL 0.05% trypan blue for 1.5 hr (Brundrett et al., 1994), vacuum-filtered through the Millipore apparatus to retain stained AMF hyphae on the nitrocellulose membrane, and rinsed by passing 1-2 mL of DI water through the filter. The nitrocellulose membranes were placed on 25 mm microscope slides and allowed to dry completely before securing cover slips with PVLG. AMF hyphal length was estimated using the gridline-intersect method (Brundrett et al., 1994) with a 10 mm\(^2\) gridded graticular eyepiece (100 squares total) at 100 x magnification and counting 50 fields of view per slide. I distinguished between AMF and non-AMF hyphae, using similar criteria as for internal hyphae (Miller et al., 1995; Mosse, 1959; Nicolson, 1959). Length of extraradical AMF hyphae within each plot was expressed as m hyphae g\(^{-1}\) soil, calculated using Tennant’s equation (Brundrett et al., 1994). Hyphal extraction efficiency was measured as described in Miller et al. (1995),
resulting in an extraction efficiency of 88%, which was applied as a correction factor to measured values.

3.2.6. Soil Aggregate Stability and Nutrients

To determine the percentage of water-stable soil aggregates in bulk soil samples, I used a wet-sieving apparatus (Eijkelkamp, Giesbeek, NL) as described in Wuddivira and Camps-Roach (2007). For each plot, a 4 g subsample of air-dried soil sieved to 2 mm was first placed into the apparatus equipped with a 250 µm sieve to retain small macroaggregates, covered with DI water, and rotary sieved in water for 3 min (stroke = 1.3 cm, approximately 34 times/min). All material washed through the 250 µm sieve was further passed through a 53 µm sieve to retain microaggregates (including those housed within unstable small macroaggregates). Material retained on each sieve was dispersed by adding a solution of 2 g L⁻¹ (NaPO₃)₆ and sieving on the apparatus for 5–8 minutes. The dispersed solutions from each sieve size and the DI water-washed material not retained on the 250 µm or 53 µm sieves (designated as not water stable, NWS) were then transferred into pre-weighed cans and dried at 105 °C for 48 hr. The percentages of water-stable soil aggregates within a 4 g subsample were calculated separately for small macroaggregates (250—2000 µm) and microaggregates (53—250 µm) by calculating the weight of soil obtained in the dispersing solution cans for each sieve size divided by the sum of the weights obtained in both of the dispersing solution cans and the distilled water can. After water-stable aggregate analysis, dried soils from small macroaggregate, microaggregate, and NWS were ball-ground. Total C and N concentrations (%) within each aggregate fraction were determined on an elemental analyzer (FlashEA 1112 series, Thermo Fisher Scientific, Waltham, MA).
3.2.7. Statistical Analysis

Significant main effects of endophyte treatment ($\alpha = 0.05$) were assessed on AMF and DSE root colonization rate, tissue N and P for roots and shoots, and percentage of water stable soil macro- and micro-aggregates using the PROC MIXED procedure in SAS (9.3 SAS Institute Inc., Cary, NC, USA) for a RCBD design with endophyte treatment as a fixed effect and block as a random effect. Averaged AMF and DSE colonization rates across individual ramets per plot were used for statistical analysis. Significant main effects of endophyte treatment and soil aggregate size fraction on %C and %N within aggregates were analyzed as a split-plot design using the PROC MIXED procedure in SAS, with endophyte treatment and aggregate size as fixed effects and block as a random effect. Significant differences between treatment means were compared using LSMEANS and the PDIFF option in SAS. Potential correlations between all quantitative parameters such as AMF colonization and plant nutrients were also assessed using the PROC REG procedure in SAS, and are reported where significant.

3.3. Results

3.3.1. Root Mycorrhizal Colonization

Overall, total AMF and DSE colonization in tall fescue roots was relatively high at the site, averaging 38 ($\pm$2) and 20 ($\pm$1) %, respectively. I found no significant effect of E. coenophiala endophyte treatment on total root AMF colonization rates (%) (Fig. 3.1A; $p = 0.5751$). I observed, however, that CTE+ plants had 7 percentage points lower AMF colonization than E− or the NTE+ treatments (33% infection for CTE+ vs. 40% averaged across other endophyte treatments). No significant effect of aboveground endophyte treatment was found on rates of AMF arbuscule, vesicle, or hypha presence ($F_{3, 15} =$
0.42—1.43; all p > 0.05) or colonization by DSE (F3, 15 = 0.10; p = 0.9586; Table 3.1).
Additionally, I observed no correlation between total AMF and DSE colonization (Regression p = 0.8313; R² = 0.0021).

3.3.2. Plant Nutrients

Endophyte treatment did not significantly influence %N (F3, 15 = 1.13; p = 0.3669) or P (F3, 15 = 1.04; p = 0.4025) in tall fescue root tissue (Fig. 3.2A,B). Nor was there a significant effect of endophyte treatment on N (F3, 15 = 2.86; p = 0.0722) or P (F3, 15 = 0.38; p = 0.7672) concentration in aboveground plant shoot tissue (Fig. 2A,B). However, the marginal trend for an endophyte effect on shoot %N was driven by a significant difference in LSMeans between CTE+ and E− (p = 0.0112, with CTE+ 0.16 percentage points > E−). I also found that P concentration in plant shoots exhibited a significant linear relationship with total root AMF colonization, where %AMF was higher in roots of plants containing lower shoot %P (Fig. 3.3). No other significant relationships were identified with nutritional parameters. In addition, there was no effect of endophyte treatment on the N:P ratio in root (F3, 15 = 0.23; p = 0.8722) or shoot (F3, 15 = 0.67; p = 0.5823) material (Table 3.2).

3.3.3. Extraradical Soil AMF

I found no significant influence of endophyte treatment on soil extraradical AMF hyphal length (m hyphae g⁻¹ soil; Fig. 3.1B; F3, 15 = 0.81; p = 0.5097). Although, unlike root colonization, plots with CTE+ tall fescue exhibited greater soil hyphal length than E− or NTE+ plots (particularly AR542 NTE+). Across endophyte treatments, plots that hosted greater total root AMF colonization contained less extraradical soil hyphae (Fig.
3.1C), but I found no significant relationships with other measured plant or soil nutritional parameters.

3.3.4. Soil Aggregate Stability and Nutrients

Endophyte treatment did not significantly influence the proportion of non-water stable aggregates (F$_{3, 15} = 1.30$; p = 0.3109), water stable microaggregates 53—250 µm (F$_{3, 15} = 0.45$; p = 0.7221), water stable small macroaggregates 250um—2000 µm (F$_{3, 15} = 0.64$; p = 0.5992), or the total amount (macro + micro) of water stable aggregates (F$_{3, 15} = 1.30$; p = 0.3109).

Carbon concentration within each soil aggregate size was determined largely by size class (aggregate size F$_{2, 40} = 45.74$; p = <0.0001), and not by endophyte treatment (Endophyte F$_{3, 15} = 1.89$; p = 0.1755) or an interaction between aggregate size and endophyte treatment (Endophyte *aggregate size F$_{6, 40} = 1.11$; p = 0.3758). Carbon concentration was highest in the small macroaggregate and NWS fractions (250 vs 53 µm p <0.0001; NWS vs 53 µm p<0.0001), but not different between the two (p = 0.3490). N concentration within each soil aggregate size was also determined by size class (aggregate size F$_{2, 40} = 116.24$; p = <0.0001), and not by endophyte treatment (Endophyte F$_{3, 15} = 0.92$; p = 0.4548) or an interaction between aggregate size and endophyte treatment (Endophyte*aggregate size F$_{6, 40} = 0.47$; p = 0.8241). Parallel to C, N concentration was also highest in the small macroaggregate and NWS fractions (LSMeans 250 vs 53 µm p <0.0001; NWS vs 53 µm p<0.0001), but did not significantly differ between the two (Table 3.3; p = 0.8391).
3.4. Discussion

To my knowledge, this is the first study quantifying aboveground symbiont *E. coenophiala* strain effects on AMF and DSE colonization in tall fescue roots and soil hyphae. Belowground root colonization by AMF and DSE and hyphal growth in soil did not differ between E−, CTE+, AR542 NTE+, or AR584 NTE+ tall fescue. There were no significant fungal symbiont associated changes in above- or belowground plant nutrients, water-stable soil aggregates, or aggregate-associated C and N, although I did note a slight negative correlation between plant shoot P and root AMF colonization, and between soil AMF hyphae and root AMF colonization. Aboveground *E. coenophiala*, regardless of alkaloid production potential or fungal strain, neither antagonizes belowground AMF and DSE in shared tall fescue hosts nor produces substantial effects on plant nutrients or soil properties in this mesic, temperate, P-rich North American pasture.

My first hypothesis, that CTE-symbiosis in tall fescue would inhibit root AMF and DSE colonization compared to NTE+ or E− plants, was unsupported by my study. This contrasts with previous demonstrations that the common toxic strain of *E. coenophiala* reduces AMF colonization in roots and inhibits AMF propagules in surrounding soils (Chu-Chou et al., 1992; Guo et al., 1992; Mack and Rudgers, 2008). This discrepancy is likely due to methodological differences in both the experimental settings and potential AMF communities between the prior experiments and this long-term field study. Prior studies were conducted in greenhouse settings for one growing season [103 d from seed in Mack and Rudgers (2008) and 15 weeks from seed in Guo et al. (1992)] and used either a live soil inoculum from nearby fields, a commercial fungal inoculum with one strain (Mack and Rudgers, 2008), or single-species isolates of *Glomus*
sp. taken from field soils (Guo et al., 1992). While Chu-Chou et al. (1992) used soil and tall fescue seeds harvested from three year-old field plots of monoculture CTE+ and E− plants, they measured propagules kg soil−1 and spores per plant using most probable number (MPN) assays described in An et al. (1990) and McGraw and Hendrix (1986). In this method, one serially dilutes a soil sample with sterilized sand, grows a host plant from seed, then harvests the soil and roots to examine for spores and other AMF propagules. Adding sand greatly facilitates root and soil preparation and analysis, yet this method essentially captures initial colonization capacity on seedlings using environmental inoculum instead of directly examining plants and soils taken from the field as in my study, which likely contributed to differences in my results.

Tall fescue harvested for this study had experienced 5 growing seasons, and only the aboveground fungal symbiont was manipulated within seeds, with no controls on belowground soil microbial communities within treatment plots. It is possible that plants harboring different aboveground symbiont strains preferentially accumulated different communities of root AMF symbionts with time, as has been demonstrated in mixed grass species plots (Vandenhoornhuyse et al., 2003). While the above studies proved that *E. coenophiala* symbiosis has the capacity to reduce root AMF colonization in tall fescue and inhibit certain AMF species in surrounding soils, I found that these differences did not always persist in the environment over the course of several growing seasons or extend to other belowground symbionts such as DSE. Research remains to be done evaluating potential effects of different endophyte strains on belowground fungal communities over time.
Neither endophyte treatment nor root symbiont colonization rates were particularly important in determining above- and belowground tall fescue nutrient concentrations. Average root and shoot N:P ratios of 4.6 and 3.9, respectively, within plant tissue denote that this site is N-limited and P-rich (Koerselman and Meuleman, 1996), which predictive theory suggests would result in a commensal relationship between plants and AMF (Johnson, 2010). I noted a weak negative correlation between total AMF colonization and grass shoot P (Fig. 3.3). This was also observed in Ryan et al. (2000), and may suggest that plants began to rely more heavily on AMF colonization as they became P-deficient in shoots. An alternative hypothesis is that, in this site’s high P soils, the optimum plant benefit from AMF is achieved at lower colonization rates, while higher colonization rates produced no additional benefit or even antagonistic feedback to plant P (Gange and Ayres, 1999). It is difficult, however, to fully evaluate AMF contribution to P uptake at different colonization levels based solely on plant P (Smith et al., 2004), so I cannot completely explain what caused the observed relationship. Still, I believe that commensalism between tall fescue and AMF at our P-rich site may have modulated nutritional contribution from AMF, which would likely have differed in more nutrient-limited conditions.

I also found no support for my second hypothesis, that plots containing CTE+ tall fescue would support less extraradical AMF hyphae in soil compared to NTE+ or E− plots. In fact, I observed a trend in the opposite direction, with a small increase in the length of extraradical soil hyphae in CTE+ plots compared to E− or the two NTE+ treatments. This contrasts with Antunes et al. (2008), who observed inhibition of inoculated root AMF colonization in Bromus inermis Leyss. (smooth brome) subjected to
decomposing CTE+ tall fescue thatch for 120 d in a glasshouse experiment, but not to AR542 NTE+ thatch, which suggested that perhaps extraradical growth through soil may have been affected in addition to spore germination or root colonization. Although lacking significant differences due to both endophyte presence and strain, my results support the alternative hypothesis proposed by Antunes et al. (2008): differences between CTE+ and NTE+ strains are potentially not due specifically to human-selected presence or amounts of livestock-toxic ergot alkaloids, but to unselected factors that differ depending on host genetics or nutrient resources, such as other alkaloids or metabolites (Rasmussen et al., 2007; Rasmussen et al., 2008), or belowground root exudates (Guo et al., 2015).

My data also contrast with those from an adjacent location containing the same tall fescue variety and endophyte treatments of a similar stand age, in which Rojas et al. (2016) found increased abundance of AMF DNA in bulk and rhizosphere soils in E+ plots compared to E− regardless of endophyte strain. My estimates of extraradical hyphal length do not reflect these findings, potentially because of different analysis methods. Uneven distribution of nuclei within aseptate AMF hyphae can unbalance analyses of DNA abundance and cause poor correlation with microscopy-based examinations (Gamper et al., 2008). Further, certain AMF species preferentially produce either spores, hyphae, or root colonization structures (Varela-Cervero et al., 2015), and AMF spores and hyphae can harbor different concentrations of nuclei (Marleau et al., 2011). That I evaluated hyphal length via microscopy and did not account for spores present in soil samples, which would have been included in DNA analyses, may explain why I did not observe the same differences as Rojas et al. (2016).
Species-specific allocation between spores, hyphae, and root colonization structures may also explain the differences observed in AMF communities between intraradical root colonizers and soil extraradical mycelium (Hempel et al., 2007). Differences in AMF species represented between roots and soils may be why plant root AMF was inversely proportional to the amount of extraradical soil AMF in this study (Fig. 3.1C). Although my findings are not statistically significant, it is possible that endophyte-mediated effects on AMF communities could manifest as tradeoffs between root colonization and soil extraradical networks, as suggested by lower root colonization rates but higher extraradical soil hyphae in CTE+ stands. These AMF-species tradeoffs could be amplified with time with continued reductions in plant diversity and higher abundance of CTE+ tall fescue compared to E− or NTE+ plots, as previously reported at this site (Iqbal et al., 2013; Slaughter et al., 2015). Future changes in AMF might be similarly reflected in DSE colonization rates or species with time, as positive associations between these symbionts have been previously observed (Ranelli et al., 2015), yet the lack of correlation between AMF and DSE in this study makes further hypotheses difficult. I propose that future studies of *Epichloë* endophyte effects on belowground symbioses both within host plants and in associated soil consider differences in fungal species.

My third hypothesis, that long-term E− or NTE+ field plots supporting higher AMF colonization rates, and likely also increased hyphae in soil, would exhibit increased percentages of water stable soil aggregates, was also not validated by the results of my study. This is likely because I found no significant changes in extraradical soil hyphae, which directly impact soil aggregate size (Miller and Jastrow, 1990). My lack of
observed changes in both water stable macro- and microaggregates and the C and N contents within aggregates suggests that previously reported increases in total soil C and N due to CTE-symbiosis within tall fescue stands (Franzluebbers et al., 1999; Iqbal et al., 2012) were not due either to differences in soil aggregation or to differences in soil C sequestered in different aggregate sizes, despite earlier findings that C and N was primarily accumulated and protected in small macroaggregates due to CTE-symbiosis (Franzluebbers and Stuedemann, 2005). However, the age of the study might also have contributed to lack of significant differences, as endophyte effects on either soil aggregates or aggregate-associated C or N are difficult to detect in short term studies [e.g., ≤ 60 weeks (Casas et al., 2011; Franzluebbers, 2006)], yet may be detectable after 20 years (Franzluebbers and Stuedemann, 2005). Previous reports of changes in total C and N were also detected after at least eight years (Franzluebbers et al., 1999) or 5-20 years (Iqbal et al., 2012). It is possible that because my analysis occurred only five years after planting, subtle increases in extraradical hyphae within CTE+ plots in the current study, even with increased abundance of CTE+ tall fescue (Iqbal et al., 2013; Slaughter et al., 2015), have not had sufficient time to significantly impact slowly-altered physical characteristics like water stable soil aggregates.

3.5. Conclusions

I found that, in a five-year old field study, neither *E. coenophiala* presence nor strain significantly impacted root AMF or DSE colonization of tall fescue or extraradical hyphae in soil. Although some differences were observed, such as decreased total root AMF colonization and increased extraradical hyphae length in CTE+ plots compared to the other endophyte treatments, these effects were subtle and did not result in any
endophyte-associated changes in plant P or N, water stable soil aggregates, or aggregate-associated C or N. My report of similar soil AMF and root AMF and DSE within two strains of NTE+ tall fescue is novel, yet the disparity of my overall results with those of prior studies examining *E. coenophiala*: tall fescue: AMF relationships call attention to the sensitivity of these tripartite interactions to other environmental parameters, such as stand age, field conditions, and AMF species. It is possible that small CTE strain-related differences in AMF colonization and soil hyphae have not yet contributed to long-term differences in plant growth or soil carbon cycling dynamics at this site. I therefore suggest that both presence and manipulation of *E. coenophiala* strains in tall fescue may not substantially alter belowground symbioses and associated plant nutrition or belowground nutrient cycling, at least in P-rich pasture ecosystems in the U.S. The high P environment of these soils may explain the largely commensal interaction I observed between aboveground *E. coenophiala* and belowground AMF, an outcome that could differ among other soils or field conditions and that should be mechanistically explored in future studies.
3.6. Tables and Figures

Table 3.1 Colonization rates (%) of arbuscular mycorrhizal fungi (AMF) arbuscules, vesicles, and hyphae, and the rate of dark septate endophyte (DSE) colonization (total % of both hyphae and microsclerotia) measured in tall fescue roots. Values are means (± S.E) of 6 replicates within each treatment.

<table>
<thead>
<tr>
<th>Endophyte Treatment</th>
<th>E−</th>
<th>CTE+</th>
<th>AR542 NTE+</th>
<th>AR584 NTE+</th>
<th>Site Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMF Arbuscules (%)</td>
<td>11 (2)</td>
<td>8 (3)</td>
<td>14 (3)</td>
<td>15 (3)</td>
<td>12 (1)</td>
</tr>
<tr>
<td>AMF Vesicles (%)</td>
<td>5 (1)</td>
<td>5 (1)</td>
<td>6 (1)</td>
<td>3 (1)</td>
<td>5 (1)</td>
</tr>
<tr>
<td>AMF Hyphae (%)</td>
<td>24 (3)</td>
<td>20 (4)</td>
<td>19 (2)</td>
<td>21 (4)</td>
<td>21 (2)</td>
</tr>
<tr>
<td>DSE colonization (%)</td>
<td>19 (3)</td>
<td>21 (2)</td>
<td>20 (3)</td>
<td>19 (3)</td>
<td>20 (1)</td>
</tr>
</tbody>
</table>

Table 3.2 Ratio of N: P in tall fescue root and shoot tissue. Values are means (± S.E.).

<table>
<thead>
<tr>
<th>Endophyte Treatment</th>
<th>N: P</th>
<th>E−</th>
<th>CTE+</th>
<th>AR542 NTE+</th>
<th>AR584 NTE+</th>
<th>Site Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot</td>
<td>3.72 (0.23)</td>
<td>4.04 (0.15)</td>
<td>4.01 (0.16)</td>
<td>3.92 (0.25)</td>
<td>3.92 (0.10)</td>
<td></td>
</tr>
<tr>
<td>Root</td>
<td>4.55 (0.28)</td>
<td>4.36 (0.53)</td>
<td>4.78 (0.25)</td>
<td>4.52 (0.30)</td>
<td>4.55 (0.17)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.3 Proportion (%) of non-water stable (NWS) silt and clay, water stable micro-aggregates (53-250 μm), and water stable small macro-aggregates (250-2000 μm) in soil samples, and aggregate-associated C and N concentration (%). Values are means (± S.E.).

<table>
<thead>
<tr>
<th>Aggregate Size</th>
<th>Endophyte Treatment</th>
<th>E−</th>
<th>CTE+</th>
<th>AR542 NTE+</th>
<th>AR584 NTE+</th>
<th>Site Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>% NWS&lt;sup&gt;A&lt;/sup&gt;</td>
<td></td>
<td>9.8</td>
<td>11.0</td>
<td>11.3</td>
<td>10.0</td>
<td>10.2</td>
</tr>
<tr>
<td>% C</td>
<td></td>
<td>2.1</td>
<td>2.1</td>
<td>2.2</td>
<td>2.3</td>
<td>2.2</td>
</tr>
<tr>
<td>% N</td>
<td></td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>% 53—250 μm&lt;sup&gt;B&lt;/sup&gt;</td>
<td></td>
<td>12.5</td>
<td>11.0</td>
<td>12.3</td>
<td>11.0</td>
<td>11.7</td>
</tr>
<tr>
<td>% C</td>
<td></td>
<td>1.7</td>
<td>1.7</td>
<td>1.9</td>
<td>1.7</td>
<td>1.8</td>
</tr>
<tr>
<td>% N</td>
<td></td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>% 250—2000μm&lt;sup&gt;A&lt;/sup&gt;</td>
<td></td>
<td>77.7</td>
<td>79.2</td>
<td>76.4</td>
<td>79.1</td>
<td>78.1</td>
</tr>
<tr>
<td>% C</td>
<td></td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
<td>2.3</td>
<td>2.2</td>
</tr>
<tr>
<td>% N</td>
<td></td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
<td>0.2</td>
</tr>
</tbody>
</table>

<sup>A</sup>, <sup>B</sup> Different letters indicate significant differences in mean C and N between soil aggregate sizes (F<sub>2,40</sub> = 45.74—116.24; all Aggregate Size p = <0.0001). Although C and N were analyzed individually, only one letter is used to indicate parallel patterns of significant differences between soil aggregate sizes for ease of interpretation.
Figure 3.1 A) Root colonization rate (%) of arbuscular mycorrhizal fungi (AMF) measured in tall fescue roots. B) Length of extraradical AMF hyphae in soil samples (m hyphae g⁻¹ dry soil). Values in panels (a) and (b) are means (± S.E.) of the 6 replicates within each treatment, while panel C) shows the linear regression of soil extraradical AMF (m hyphae g⁻¹ dry soil) in soil samples with tall fescue root AMF colonization (%) across the 24 research plots labeled by endophyte treatment.
Figure 3.2 A) N and B) P concentration (%) in shoot and root tall fescue tissue. Values are means (± S.E.) of each treatment.
Figure 3.3 Linear regression of shoot P concentration (%) and root AMF colonization (%) in tall fescue.

Regression $p = 0.0126$; $R^2 = 0.2512$
Chapter Four

Tall fescue-\textit{Epichloë coenophiala} associations affect belowground fungi and host, symbiont response to climate change

4.1. Introduction

Plants frequently host both above and belowground symbioses with microorganisms, the interactive nature of which can range along a continuum from parasitic or pathogenic, which harm or impair plant function, to mutualistic, where microorganisms provide beneficial services for the host plant such as enhanced nutrient acquisition and increased productivity (Bronstein, 1994; Carroll, 1988; Johnson et al., 1997). One important plant-microbe symbiosis found in both agronomic and natural grasslands worldwide occurs between cool-season grass species and asexual fungal endophytes of the genus \textit{Epichloë}, whose hyphae intercellularly inhabit aboveground grass stems and leaves and are transmitted vertically between plant host and progeny within seeds (Schat et al., 2004). In pastures of North America, for example, tall fescue (\textit{Schedonorus arundinaceus} Schreb.) is an important perennial forage grass covering about 15 million hectares across the U.S. (Rogers and Locke, 2013), much of which forms a host-specific association with \textit{Epichloë coenophiala} (Leuchtmann et al., 2014; Shelby and Dalrymple, 1987).

The nature of this association is commonly considered a defensive mutualism (Clay, 1988), whereby \textit{E. coenophiala} provides alkaloid compounds that deter mammalian and insect herbivory, while also increasing drought tolerance (Arachevaleta et al., 1989; Bouton et al., 1993; Elmi and West, 1995) and competitive ability (Hill et al., 1991) of tall fescue in exchange for photosynthetically-produced C and other apoplastic
compounds, shelter, and dispersal through seed. However, ergot alkaloids produced by common toxic strains of *E. coenophiala* (CTE) are well-known to harm grazing livestock animals, reducing bodyweight gain and reproductive rates (Strickland et al., 2011).

Non-livestock-toxic or ‘novel’ toxic endophyte (NTE) strains of *E. coenophiala*, which do not produce the alkaloids that harm livestock yet still produce insect-deterring alkaloids, have been isolated from wild populations and introduced into elite tall fescue forage varieties (Bouton et al., 2002; Hopkins et al., 2011). In addition to attempts to improve E− tall fescue (Bouton et al., 2001), varieties containing NTE strains are continually being created and marketed as replacements of CTE+ tall fescue [e.g., (Hill et al., 2010; Hopkins et al., 2011)]. Although the effects of common toxic endophyte symbiosis, or infection, (CTE+) on tall fescue and plant communities have been thoroughly examined [e.g., (Rudgers and Clay, 2007)], we are only recently investigating the potentially different ecosystem impacts of novel toxic endophyte-symbiotic (NTE+) tall fescue [e.g., (Rudgers et al., 2010)].

Within most terrestrial plants, including grasses such as tall fescue, arbuscular mycorrhizal fungi (AMF) of the phylum *Glomeromycota* are another widespread fungal symbiont that colonize belowground roots and are best-known for their nutritionally mutualistic effects on hosts and associated impacts on ecosystem processes (Schüßler et al., 2001; Smith and Read, 2008). AMF can provide increased water and nutrient uptake in host plants (Augé, 2001), help increase soil aggregate size and stability and ecosystem C sequestration (Duchicela et al., 2013; Miller and Jastrow, 1990; Wilson et al., 2009), and may even play a role in pest, pathogen, and allelopathic chemical resistance in host plants (Abhiniti et al., 2013; Barto et al., 2010; Sikes et al., 2009; Tao et al., 2016). Yet,
the nature of this symbiosis may not always be mutualistic, and is governed by a hierarchy of biotic and abiotic factors at multiple ecosystem scales, such as the availability of P and N that AMF frequently provide to the plant (Johnson et al., 1997). In addition, other belowground fungal symbionts are found alongside AMF. These include dark septate endophytes (DSE) of the phylum *Ascomycota*, which may exhibit similar function to AMF (Mandyam and Jumpponen, 2005; Mayerhofer et al., 2013; Newsham, 2011; Wagg et al., 2008). How the roles and activities of these belowground fungi are altered by factors such as host plant genetics and environmental conditions, and the resulting impacts on ecosystem productivity and processes, remains to be fully understood.

It also remains unclear how these belowground fungal symbionts interact with other plant-associated microbes or aboveground symbionts, such as *E. coenophiala*. Studies have demonstrated that aboveground CTE-symbiosis inhibits AMF colonization rates in tall fescue roots and AMF structures or abundance in soil (Buyer et al., 2011; Chu-Chou et al., 1992; Guo et al., 1992; Mack and Rudgers, 2008). Further, presence of CTE+ tall fescue litter can inhibit AMF colonization in other plants such as smooth brome grass, yet this effect is not observed when using endophyte-free (E−) or NTE+ litter (Antunes et al., 2008). Similarly, symbiosis with livestock-toxic *E. festucae* var. *lolii* decreased concomitant AMF colonization in perennial ryegrass (Müller, 2003). While protective ergot alkaloid compounds produced only by CTE strains may therefore be responsible for these effects, it is also possible that other factors influence these interactions such as differences in host or symbiont genetics, additional non-ergot
alkaloids or metabolites (Rasmussen et al., 2007; Rasmussen et al., 2008), or belowground root exudates (Guo et al., 2015).

In contrast, other *Epichloë* endophytes have been shown to increase AMF colonization within their plant hosts (Novas et al., 2005; Novas et al., 2009; Novas et al., 2011) and additively increase plant growth (Larimer et al., 2012; Novas et al., 2005). *Epichloë occultans* symbiosis in annual ryegrass has been further shown to stimulate AMF in neighboring *E−* plants, despite decreased AMF colonization within shared hosts (Omacini et al., 2006). Liu et al. (2011) found that availability of host resources such as plant C may help mediate these interactions, demonstrating that *E. festucae var. lolii* decreased belowground AMF colonization in one cultivar of perennial ryegrass but not in a higher-sugar producing cultivar.

In Chapter 3, I found that neither presence nor strain (one CTE, two NTE) of *E. coenophiala* significantly altered belowground AMF colonization in tall fescue roots collected from five-year old field plots. This body of work suggests that environmental factors such as high P, abundant rainfall, advancing plant age, and accrual of distinct AMF communities, may influence these tripartite interactions and potentially alleviate antagonistic behavior between symbionts. However, we do not fully understand how these and other biotic or abiotic factors interactively govern relationships between plant symbionts or their roles within the plant, or how alterations in one or more influential factors potentially impact these symbioses and associated ecosystem consequences.

Plant-microbe symbioses may be especially important in governing ecosystem responses to climate change (Compant et al., 2010; Kivlin et al., 2013). Yet, how plant-microbe interactions within grassland ecosystems may respond to future climatic
alteration is still poorly understood. For example, studies investigating the endophyte-associated increase in drought tolerance or response to other temperature or water stress in tall fescue are highly variable, with a range of studies observing positive (Arachevaleta et al., 1989; Elmi and West, 1995), negative (Assuero et al., 2000; Hill et al., 1996), or neutral (Elbersen and West, 1996; MacLean et al., 1993) effects, which may be due to genotypic differences in tall fescue x endophyte associations [e.g., (Assuero et al., 2000; Elbersen and West, 1996; Malinowski et al., 2000; Yurkonis et al., 2014)]. A recent study by Bourguignon et al. (2015) utilizing different tall fescue-endophyte combinations found that tall fescue’s response to climate change varied depending on host and endophyte genetics and was more sensitive to warming than increased precipitation. As host-endophyte responses to climate change vary with genetics, so too may their impact on other plant-microbe symbioses such as AMF colonization.

While few studies to date have investigated the effect of both \textit{E. coenophiala} symbiosis in tall fescue and predicted climate change factors on AMF, some studies of AMF in grasses have shown increased root colonization rates in response to warming (Bunn et al., 2009; Büscher et al., 2012; Kim et al., 2014; Rillig et al., 2002). Colonization by DSE has also been shown to increase with warming (Olsrud et al., 2010). Conversely, Heinemeyer and Fitter (2004) found warming-induced increases in extraradical AMF mycelium extending from two of three tested plant species, but produced no effects on root colonization. Yang et al. (2013) further discovered that warming significantly influenced AMF community composition in soils, but not roots. These results show that different AMF structures and species may respond differently to altered temperatures. Heinemeyer et al. (2004) found little effect of soil warming, but
observed a reductive effect of shading on root AMF colonization, suggesting that photosynthetic resources limit AMF responses to climate.

Reports of altered moisture regime effects on AMF colonization are mixed, such as Owens et al. (2012) finding no effects of altered precipitation on AMF colonization in two warm season grasses, while Cavagnaro (2016) observed that soil moisture restrictions prior to planting increased AMF colonization and mycorrhizal responsiveness in tomato. Long term summer drought can increase root AMF colonization while decreasing extraradical hyphae (Staddon et al., 2003). The effects of climate alteration on belowground fungal colonization are complex, and few researchers have further investigated how multiple simultaneous plant-microbe symbioses or plant and fungal genetics interactively govern these responses. Ascertaining how multiple symbionts, such as aboveground *Epichloë* endophytes and belowground AMF, and genetic variants interact in coordination with global change factors will be key to understanding and predicting ecosystem response to future conditions (Kivlin et al., 2013).

To address this knowledge gap, I used an established long term manipulative field climate change study to: 1) Examine host genotype and endophyte symbiosis controls on root colonization by belowground fungi and associated plant nutrient concentrations and soil properties; 2) Discover how warming and/or added growing season precipitation altered these relationships. I hypothesized that: 1) Physiological differences between unique combinations of host and endophyte genotypes would differentially affect root colonization by belowground AMF and DSE and associated plant and soil properties, such as lower defensive alkaloid production in NTE+ tall fescue enabling greater root AMF and DSE colonization compared to CTE+ genotypes; 2) These associations would
be further impacted by warming and added growing season precipitation, such as added precipitation ameliorating the beneficial effects of root AMF colonization and thus exhibiting less root colonization compared to warmed plots, while warming may stimulate extraradical AMF hyphae in soil.

4.2. Materials and Methods

4.2.1. Site Description and Study Design

The study was located at the University of Kentucky Spindletop Research Farm in Lexington, Kentucky (38°06’29.24”N; 84°29’29.72”W). This site is 281 m above sea level, receives approximately 1163 mm annual precipitation (30-yr mean), and experiences mean annual summer and winter temperatures of 23.8°C and 1.6°C, respectively (Ferreira et al., 2010). The underlying soil was a Bluegrass-Maury silt loam complex with approximately a 2% slope, which is a well-drained, fine-silty, mixed, active, mesic Typic Paleudalf that formed from silty non-calcareous loess over clayey residuum derived from phosphatic limestone (Soil Survey Staff et al., 2014). In spring 2008, the experimental pasture was prepared and seeded with Kentucky bluegrass (*Poa pratensis* L.), tall fescue (*Schedonorus arundinaceus* Schreb.), red clover (*Trifolium pratense* L.), and white clover (*Trifolium repens* L.). In August 2008, bermudagrass (*Cynodon dactylon* L. Pers.) sprigs taken from a nearby established pasture were plugged across the experimental area [see (Brosi, 2011)].

In 2009, five experimental blocks were established in the existing pasture. Each block consisted of four, 5.8 m² hexagonal plots, each containing one of the following climate treatments: 1) +Heat (+3°C, year-round); 2) +Precip (+30% of long-term annual mean precipitation, added in growing season); 3) a combination of +Heat+Precip; 4)
Control (ambient: 0 Heat, 0 Precip) conditions. Climate treatments were applied continuously from 1 May 2009 until 15 November 2013.

Temperatures 3 °C above ambient conditions were constantly and uniformly maintained in the warmed plots (+Heat, +Heat+Precip) using 1000 W Salamander infrared heaters (Mor Electric Heating Assoc., Comstock Park, MI) located around the edges of each plot (Kimball et al., 2008). These were maintained at 120-cm above the plant canopy and angled at 45° toward the ground at the center of each plot. To account for possible shading effects of the heating units, ambient temperature plots (+Precip, Control) were surrounded by heater housing units that lacked infrared heaters.

In treatment plots receiving added precipitation (+Precip and +Heat+Precip), rainwater collected on site and stored in a water tank was applied using metered wands throughout the growing season (April – September), primarily during precipitation events (2 per month). The total amount applied during the growing season was calculated, using long term monthly trends, to equal 30% of the long-term mean precipitation. During study establishment, aluminum flashing was installed to a depth of 50 cm surrounding each of the study plots to prevent lateral water movement between treatments.

Air temperature, soil temperature, and soil moisture were continuously monitored in each plot between 1 May 2009 and 15 November 2013 to determine efficiency and consistency of climate manipulations. Every 15 minutes, thermocouples at a depth of 5 cm recorded soil temperature measurements, while time domain reflectrometer (TDR) probes recorded volumetric water content (0—15 cm). Beginning on 1 June 2009, all plant biomass above 7.6 cm from the soil surface was mowed and removed from the site three times per year (in May, July, and October).
In each of the 20 climate treatment plots, plant genetic clone pairs (n=4), where one individual of each clone was endophyte-infected and the other individual was endophyte-free, were planted on 25 October 2011 (n=8 individuals planted per plot). These were planted in a 30 x 60 cm area divided equally into 8 square sub-plots (one per plant). As described in (Bourguignon et al., 2015), clones were chosen from an assemblage of material developed in 2006 by T. Phillips and R. Dinkins, which originated from locally harvested common toxic E. coenophiala endophyte-infected tall fescue (KY-31 variety) seeds. Half of the tillers from each individual plant was treated with Folicur 3.6F fungicide (Bayer Crop Science, Monheim, Germany) [tebuconazole (1-[4-chlorophenyl]-4,4-dimethyl-3-[1,2,4-triazol-1-ylmethyl]pentan-3-ol)] to remove the common toxic endophyte and create an endophyte-free tall fescue clone as described (Nagabhyru et al., 2013). Each individual thus became a genetic clone pair that contained endophyte infected (CTE+) and endophyte free (E−) material. In 2008, the clone pairs were moved from greenhouse pots into a field site at Spindletop farm. Also in 2008, KY-31 tall fescue seeds infected with novel endophyte strains that were acquired from Morocco by Dr. Charles West, then at the University of Arkansas, were put through a similar procedure to create NTE+ and E− clones. The present study utilized E+ and E− clone pairs of two tall fescue genotypes whose E+ clones associated with common toxic endophyte strains (CTE14+ and CTE45+), and two tall fescue genotypes whose E+ clones associated with novel endophyte strains (NTE19+ and NTE16+).

4.2.2. Sample Harvest and Preparation

In October 2013, after two years of growth, each clone pair was harvested in entirety (whole plants were comprised of one to 10 tillers aboveground; harvest included
aboveground material, roots, and associated rhizosphere soil) and stored at −20 °C. Between planting in October 2011 and harvest in October 2013, 40 of the 160 original clone pairs had died, resulting in 120 total harvested plants. Additionally, ten of the 120 harvested plants were removed from statistical analyses because either endophyte infection status (+ or −), or endophyte strain, did not match the assigned treatment (Section 4.2.3). Thus, 110 plants were used for statistical analyses in this study.

Before assessing belowground AMF colonization, aboveground plant material was separated from belowground roots. Associated soil was brushed from the roots of each harvested clone pair, sieved to 2 mm, and air-dried. Belowground root material was washed, then dried for 2 d at 55 °C and weighed. After root mycorrhizal analysis, the remaining material was ball-ground for nutrient analyses. Aboveground whole plant (shoot) material was dried for 2 d at 55 °C, separated into live (green) and senesced/dead (brown) portions to record weights, and then recombined to ball-grind for nutrient analyses. Both root and shoot weights were corrected for ash content using subsamples of ball ground material combusted at 525 °C for 4 hr, and ash-corrected root and total shoot weights are reported.

4.2.3. Verification of Endophyte Presence and Strain

Molecular biochemical analyses were conducted on aboveground pseudostem material of one tiller selected from each clone pair to identify the distinct genetic profile of *E. coenophiala* strains associated with each plant as described in Takach and Young (2014). First, I extracted total DNA (unquantified) from a 0.5 to 1 cm portion of the bottom of each tiller using a MagAttract 96 DNA plant core kit (Qiagen). I amplified fungal genes from 3 µl of total DNA using primers for mating type, housekeeping, and
alkaloid biosynthesis genes (11 total) included across three multiplex PCR reactions. PCR amplicons were imaged using agarose gel electrophoresis. Based on the presence or absence of the 11 fungal genes, I was able to screen each plant for endophyte presence and distinguish between the distinct endophyte strains associated with each tall fescue genotype to ensure that harvested plants contained the correct endophyte status and strain associated with their assigned symbiotic genotype clone pair (Table 4.1).

Of the 120 harvested plants, 11 exhibited fungal genetic profiles that conflicted with the assigned treatment in terms of both fungal presence/absence and *E. coenophiala* strain, and were excluded from statistical analyses. Of these, five plants that were supposed to be E− clones were not only revealed to be E+, but contained an *E. coenophiala* strain matching the wrong genetic profile for the assigned clone pair (e.g., harvested from plot area assigned to E− clone of symbiotic genotype NTE19, but contained *E. coenophiala* profile 1, which is associated with CTE14). One plant harvested from a plot area assigned to the E+ clone of symbiotic genotype CTE45 instead contained *E. coenophiala* profile 1, which is associated with CTE14. Two plants harvested from plot areas assigned to E− clones were revealed to be E+, yet contained the *E. coenophiala* profile associated with their respective E+ clones. Lastly, three plants that should have been E+ clones instead contained no fungal genes, and were thus E−.

All 11 plants were further assessed for presence or absence of *E. coenophiala* hyphae within stained leaf sheath epidermal peels (Clark et al., 1983), although this test does not allow for differentiation between endophyte strains. Leaf peel results of only one of the 11 questionable plants conflicted with genetic screening results; this plant was designated E− and contained E+ genes of the wrong endophyte strain, yet was found to
contain no endophyte hyphae during leaf peel analysis. In accordance with leaf peel results, this sample was added back into the data pool for statistical analyses. Therefore, 10 of the 120 harvested plants were rejected from statistical analyses in this study.

4.2.4. Root Mycorrhizal and DSE Colonization

As described in Chapter 3, I assessed belowground fungal colonization in dried root subsamples from each harvested tall fescue plant using trypan blue staining and microscopy (McGonigle et al., 1990). After rehydrating roots overnight in H₂O, then clearing cellular pigments with KOH and acidifying with HCl, I stained roots with 0.05% trypan blue. Stained roots were stored in 1:1 glycerol: H₂O de-staining solution before arranging and preserving on microscope slides for subsequent microscopy. I viewed both trypan-stained AMF (arbuscules, vesicles, hyphae) and melanized DSE (microsclerotia, hyphae) structures at 400 x magnification using the line intersect method with 30 intersections modified from (McGonigle et al., 1990), recording presence of AMF and DSE separately within each view. Values are expressed as fungal colonization rates (%; number of presences divided by 30 total views, then multiplied by 100).

4.2.5 Plant C, N, and P Concentration

Ball-ground subsamples of tall fescue root and shoot material from each harvested plant were analyzed for C, N, and P concentration. Carbon and N concentrations (%) were measured via combustion on an elemental analyzer (FlashEA 1112 series, Thermo Fisher Scientific, Waltham, MA). Total N and P (%) were further assessed via digestion, in which total plant N was converted to NH₃ (Bradstreet, 1965), while total plant P was converted to PO₄³⁻. Nitrogen and P concentrations (%) were determined colorimetrically
as modified from Chaney and Marbach (1962) and Fiske and Subbarow (1925), respectively.

In the proceeding sections I always report N % obtained from combustion analysis rather than digestion, except for when calculating N:P ratios in root and shoot tissue (to be consistent with P % obtained through digestion). However, I note that N % obtained through combustion was highly correlated with N % obtained through digestion (Regression p < 0.0001, R² = 0.86 in roots; Regression p < 0.0001, R² = 0.97 in shoots).

4.2.6. Extraradical AMF in Soil

As described in Chapter 3, I extracted extraradical AMF hyphae (ERH) from soil samples using methods modified from Jakobsen et al. (1992) and Rillig et al. (1999). I used 4 g subsamples of soil associated with the roots of each harvested tall fescue plant, dispersed in a solution of H₂O and (NaPO₃)₆ and sonicated to break up soil particles and hyphae. Hyphae, roots, and organic material were retained on a 38µm sieve, then suspended in 200 mL of H₂O. Material within a 4 mL aliquot was then stained with 0.05% trypan blue for 1.5 hr (Brundrett et al., 1994) before vacuum-filtering the solution through a 0.45 µm nitrocellulose membrane in a 25 mm Millipore filter holder and passing 1–2 mL of DI water through the filter to rinse unabsorbed trypan. Dried membrane filters were preserved, covered, on 25 mm microscope slides using PVLG to await microscopy. I estimated the length of trypan-stained, non-septate or non-regularly septate extraradical AMF hyphae at 100x magnification within 50 fields of view on each slide using the gridline-intersect method described in (Brundrett et al., 1994), with a 10 mm² gridded graticular eyepiece (100 squares total). Values for extraradical AMF hyphae length in soil samples were calculated using Tennant’s equation and expressed as m
hyphae g⁻¹ soil (Brundrett et al., 1994). The efficiency of my extraction was 89%,
determined as in Miller et al. (1995), which was applied to measured values as a
correction factor.

4.2.7. Statistical Analysis

This study incorporated a split-split design within the established climate field
study. The whole plot climate treatment factor (four treatments: factorial combination of
added Heat and Precipitation), was split first by tall fescue symbiotic genotype (CTE14,
CTE45, NTE16, or NTE19) established within each climate treatment plot, and split
again by endophyte presence (E+) or absence (E−) within cloned pairs of each tall fescue
symbiotic genotype. Due to the subsequent death of some individuals (120 of the original
160 study plants remained at harvest), and samples that failed endophyte screening (10
plants further excluded for not matching assigned endophyte treatment), I was unable to
analyze data using the full factorial design at all levels of my whole- and split-plot factors
described above. Instead, I statistically analyzed my data using two approaches based on
which treatments contained a sufficient number of replicates for analyses (Table 4.2).

In Analysis 1, I excluded the climate factor levels +Heat and +Heat+Precip and
considered only Control and +Precip climate treatments. I analyzed these data for
significant fixed effects (α = 0.05) of added precipitation (Precip), symbiotic genotype
(TFtype; all 4 included), and endophyte status (Estatus) using PROC GLIMMIX in SAS
(9.3 SAS Institute Inc., Cary, NC, USA). Full tables of ANOVA results from this analysis
are included as Appendices 1A—1D.

In Analysis 2, I excluded the two symbiotic genotypes that had the fewest
replicates remaining, CTE14 and NTE16, and considered only CTE45 and NTE19 within
the full climate treatment factorial. I analyzed these data for significant fixed effects (\(\alpha = 0.05\)) of added heat and precipitation (Heat and Precip, respectively), symbiotic genotype (Tftype; only CTE14 and NTE16 included), and endophyte status (Estatus) using PROC GLIMMIX in SAS. Full tables of ANOVA results from this analysis are included as Appendices 2A—2D.

Within both of these analyses, I arcsine-transformed three measured fungal parameters (Arbuscule and Vesicle colonization, DSE colonization) and log-transformed two of the biomass measurements (Green shoot weight and Total shoot weight) to meet statistical assumptions of normality. Statistical analyses were conducted (and p-values reported) on transformed data of these parameters, but untransformed values are shown in tables and figures to retain biological relevance. For significant interactions detected in both of these analyses, I used differences in the Least Squares Means (LSMEANS, /pdiff) to determine significant differences between means (\(\alpha = 0.05\)). Using all 110 samples for statistical analyses, I also employed PROC REG in SAS to evaluate linear relationships between quantitative fungal and plant parameters, after assessing potential for linear correlations between all measured study variables using Pearson’s r in SAS (PROC CORR). Pearson’s r-values between fungal and plant parameters are included in Appendix 3, and relationships assessed via regression are reported where significant.
4.3. Results

4.3.1. Temperature and Moisture

Climate change treatments effectively altered soil moisture and soil and air temperatures at this study site (Fig. 4.2A,B,C). For example, monthly average soil volumetric water content was between 0.03 (minimum difference, January) and 0.11 (maximum difference, June) units higher during the year in +Precip plots compared to +Heat plots (Fig. 4.2A). Soil temperatures in +Heat and +Heat+Precip plots were, on average, approximately 2.5 °C higher than in Control and +Precip plots throughout (Fig. 4.2B). Similarly, air temperatures in +Heat and +Heat+Precip plots were, on average, approximately 3.2 °C higher than in Control and +Precip plots throughout the two year period (Fig. 4.1C).

4.3.2. Belowground Fungi

When assessing endophyte status and all tall fescue symbiotic genotypes among the two mesic climate treatments, Control and +Precip, (Analysis 1), I found that neither endophyte status nor tall fescue genotype significantly influenced the total rate of arbuscular mycorrhizal fungi (AMF) colonization (arbuscules + vesicles + hyphae) in tall fescue roots (Appendix 1A). However, when averaged across both E− and E+ individuals, tall fescue symbiotic genotypes CTE14 and CTE45 expressed different colonization rates of AMF arbuscules, with CTE14 containing over twice the arbuscule colonization rate of CTE45 (Table 4.3, Appendix 1A). In addition, the rate of AMF vesicle colonization was significantly higher in E− compared to E+ individuals across all tall fescue genotypes (3.28 % ± 0.67 S.E. in E− vs. 1.71 % ± 0.52 S.E. in E+; Estatus $F_{1,20} = 6.7, p = 0.0176$). The length of extraradical AMF hyphae (ERH) in root-associated
soils was also influenced by both tall fescue genotype and endophyte status (Fig. 4.3A). ERH was significantly reduced in E− clones of NTE16 compared to E− clones of either CTE45 or NTE19 (Fig. 4.3A). In addition, E− clones of NTE19 supported approximately 18 m hyphae g−1 soil more than E+ clones (Fig. 4.3A), whereas E+ and E− levels were similar for the other symbiotic genotypes. The rate of root colonization by dark septate endophytes (DSE) was not affected by tall fescue genotype, endophyte presence, or added precipitation (Appendix 1A).

When evaluating the effects of all factorial combinations of added heat and precipitation, endophyte status, and symbiotic genotype of only CTE45 and NTE19 (Analysis 2), I found no significant main or interactive influences of the factors on the total rate of AMF colonization (arbuscules + vesicles + hyphae) in tall fescue roots (Appendix 2A). Yet, endophyte presence significantly decreased the rate of arbuscule formation under ambient conditions but produced no such effect with added precipitation (Fig. 4.4). The rate of vesicle formation was interactively influenced by tall fescue symbiotic genotype, endophyte status, and added heat (Fig. 4.5A). No significant heat or endophyte-related differences were observed with CTE45 tall fescue clones. Yet, warming significantly increased vesicle % compared to ambient conditions in E+ clones of NTE19, and the opposite effect was found in E− clones of NTE19 (Fig. 4.5A).

ERH was interactively influenced by tall fescue symbiotic genotype, endophyte status, added heat, and added precipitation (Fig. 4.6). In E+ clones of CTE45, the addition of both heat and precipitation significantly increased ERH by approximately 31.1 m hyphae g−1 soil compared to ambient conditions and precipitation alone (average 72.1 m hyphae g−1 soil in Control and +Precip treatments vs. 103.2 in +Heat+Precip), but no
significant differences were observed amongst E− clones of CTE45 (Fig. 4.6). In E− clones of NTE19, the +Heat+Precip treatment supported nearly twice as much ERH as those from other climate treatments (183 m hyphae g\(^{-1}\) soil in +Heat+Precip vs. 91, on average, in Control, +Heat, and +Precip; Fig 4.6), although replication was low. Yet, in E+ clones of NTE19, the greatest difference in ERH (approximately 28.2 m hyphae g\(^{-1}\) soil) was observed between +Heat and +Heat+Precip treatments (Fig 4.6).

Root colonization by dark septate endophytes (DSE) exhibited significant main effects of both endophyte status and heat. DSE colonization of E− plants across both tall fescue genotypes and all climate treatments was approximately 6 percentage points lower than in E+ plants (12% in E− vs. 18% in E+), whereas warming alone more than doubled the rate of DSE colonization across both tall fescue genotypes and endophyte status compared to ambient conditions (Table 4.4, Appendix 2A).

4.3.3. Plant nutrients and Biomass

Within only the mesic Control and +Precip climate treatments (Analysis 1), I found that differences in tall fescue symbiotic genotypes, regardless of endophyte status, significantly impacted N %, N:P ratio and C:N ratio of tall fescue shoot tissue. Shoot N and N:P were significantly higher in symbiotic genotypes NTE16 and NTE19 compared to CTE14 and CTE45 (Table 4.5, Appendix 1B). Shoot C:N was highest in CTE14 and lowest in NTE19, yet also significantly differed between CTE14 vs. CTE45 and between NTE16 vs. NTE19 (Table 4.5, Appendix 1B). In addition, endophyte presence across all tall fescue symbiotic genotypes significantly stimulated shoot P % and root weight, while simultaneously decreasing both shoot and root N:P ratios in tall fescue tissue (Table 4.6. Appendix 1B,C,D).
Both tall fescue symbiotic genotype and endophyte status interactively influenced C:N ratios of tall fescue roots and the amount of dead tall fescue shoot tissue (Fig. 4.3B,C). Specifically, tall fescue root C:N ratios were approximately 6 points less within E+ clones compared to E− clones of NTE16, but other genotypes had no differences between E+ and E− material (Fig. 4.3B). In addition, although dead shoot tissue weight was 3.6-fold higher in E+ clones than in E− clones of CTE14 (3.60 g in E+ clones vs. 0.99 g in E− clones; Fig. 4.3C), no differences were observed for this parameter for the other symbiotic pairs. Endophyte status and added precipitation interactively influenced both dead shoot tissue weight and total shoot weight across all tall fescue symbiotic genotypes, where absence of endophyte significantly decreased both parameters when subjected to added precipitation compared to E+ tall fescue (Fig. 4.7A,B). Lastly, the amount of green shoot tissue was significantly influenced by the interactive effects of tall fescue symbiotic genotype, endophyte status, and added precipitation. In CTE14, endophyte presence (E+) significantly stimulated green shoot weight compared to the respective E− clones, regardless of precipitation treatment (Fig 4.8). However, in NTE16, added precipitation resulted in significantly lower green shoot weight in E− clones compared to ambient conditions and had no effect on E+ material (Fig. 4.8).

Within all factorial combinations of added heat and precipitation for both E− and E+ clones of tall fescue symbiotic genotypes CTE45 and NTE19 (Analysis 2), four of the measured plant nutrient and biomass parameters had a significant main effect of tall fescue symbiotic genotype (Table 4.7, Appendix 2B,C,D). Concentrations (%) of shoot N and root P were significantly higher in NTE19 tall fescue compared to CTE45, and root C:N and green shoot weights were significantly higher in CTE45 compared to NTE19
Further, live green and dead weights from tall fescue shoots were significantly higher in E+ clones across all climate treatments and tall fescue genotypes (Table 4.8, Appendix 2D). Across all climate treatments and endophyte status, five plant nutrient parameters also had a significant main effect of added heat (Table 4.9, Appendix 2B,C). Shoot N, root P, and root C:N in tall fescue all decreased with warming alone compared to ambient conditions, whereas shoot N:P, root N:P, and root C increased due to warming alone (Table 4.9, Appendix 2B,C).

Warming interacted with tall fescue symbiotic genotype to determine shoot P (%) and root N (%) irrespective of endophyte symbiosis (Fig. 4.9A,B). Warming significantly decreased shoot P compared to ambient conditions for NTE19, but this effect was not observed in CTE45 (Fig. 4.9A). In contrast, warming significantly increased root N in both CTE45 and NTE19, but to a greater degree in NTE19 (Fig. 4.9B). Weight of root and total shoot biomass across all climate treatments were interactively affected by tall fescue symbiotic genotype and endophyte status. In particular, E− clones of NTE19 had significantly lower root and total shoot weight than E+ clones (−62% of E+ root weight, −56% of E+ shoot weight), but CTE45 root and shoot weights were similar between E− and E+ clones (Fig. 4.10 A,B). Further, shoot C:N ratio, green shoot weight, and total shoot weight of tall fescue across both genotypes and endophytes statuses were interactively affected by added heat and precipitation (Fig. 4.11A,B,C). Warming and added precipitation together significantly reduced green shoot and total shoot weights compared to added precipitation alone (Fig. 4.11B,C). In the interaction between heat and precipitation, added heat alone significantly reduced shoot C:N compared all other climate treatments (Fig. 4.11A), yet shoot C:N was also influenced by a significant
interaction between tall fescue symbiotic genotype, endophyte status, and heat (Fig. 4.5B). In CTE45, warming significantly reduced shoot C:N in E+ clones compared to in ambient conditions, yet this effect was not observed in E+ clones of NTE19 (Fig. 4.5B).

Finally, tall fescue shoot P (%), shoot N:P, and shoot C:N were significantly influenced by the interactive effect of tall fescue symbiotic genotype, endophyte status, and added precipitation (Fig. 4.12A,B,C). In NTE19, additional precipitation reduced shoot P in E+ clones, but this effect was not observed in E− clones nor in any CTE45 material (Fig 4.12A). Simultaneously, added precipitation significantly increased shoot N:P in E+ clones of NTE19, but decreased shoot N:P in E+ clones of CTE45 (Fig. 4.12B). The interactive effect of tall fescue genotype, endophyte status, and precipitation on shoot C:N appeared to be driven by significant differences between genotypes. NTE19 exhibited lower shoot C:N than CTE45 in both E− and E+ clones, although this effect was only significant in ambient conditions (Fig 4.12C).

4.3.4. Soil Nutrients

In soils associated with tall fescue roots, I found no significant effects of endophyte status, tall fescue genotype, or added precipitation on soil C or N (%) in Analysis 1 (Appendix 1D). However, I found a significant interactive effect of added heat and endophyte status on soil N (%) across both tall fescue genotypes in Analysis 2 (Fig. 4.13). When subjected to warming, soils from E+ tall fescue exhibited a significantly higher N concentration compared to soils from E− tall fescue, yet this endophyte effect was not observed under ambient conditions (Fig. 4.13).
4.4. Discussion

In this study I investigated how genotypic variation in tall fescue-\textit{E. coenophiala} associations impacted belowground fungal colonization and host-symbiont response to climate change. Tall fescue symbiotic genotype and aboveground endophyte status impacted the frequency of occurrence of specific AMF structures. Because AMF structures are thought to perform different roles (Smith and Read, 2008), these results suggest that host genetics and interactions with aboveground symbionts were influencing AMF function. In addition, these genotype and aboveground endophyte controls on belowground fungi were altered when subjected to predicted climate change factors such as warming and added precipitation. For example, higher temperature appeared to make the endophyte-AMF relationship more antagonistic, as the occurrence of stress-induced AMF vesicles increased in E+ material under elevated heat, but under more mesic conditions, E+ plants had reduced occurrence of AMF vesicles. Conversely, root colonization by dark septate endophytes (DSE) was not subject to either plant or \textit{E. coenophiala} genetic control, even under warming, though both warming and endophyte presence exerted strong controls on this group of belowground symbionts. My results show that not only do host-symbiont genetic variability and aboveground endophyte presence regulate functioning of belowground AMF, but these interactions can be different under predicted future climate change conditions, which may have important implications for ecosystem response to global change.

4.4.1. Analysis 1: Genetic and endophyte controls on belowground fungi

Because \textit{E. coenophiala} and tall fescue genotype may differentially impact signaling and carbon allocation to belowground symbionts, I hypothesized that different
*E. coenophiala* and tall fescue genotypic combinations would elicit different belowground colonization rates by AMF and DSE. However, this was only partially supported. The occurrence of AMF arbuscules was strongly controlled by tall fescue genotype; CTE45 exhibited 4–10% less arbuscule colonization than the other three genotypes, regardless of endophyte symbiosis (Table 4.3). Similarly, endophyte symbiosis significantly reduced the length of extraradical AMF hyphae in soils associated with NTE19, but not in the other three genotypes (Fig. 4.3A). Yet, endophyte and tall fescue genotype had little influence on AMF vesicles, though endophyte presence significantly reduced the occurrence of AMF vesicles overall.

Arbuscules are the primary nutrient-transfer interface between host and AMF symbiont, where exchange of photosynthetically-produced plant C for nutrients acquired by AMF, such as N and P, occurs (Smith and Smith, 1989), and are considered a sign of vitality and active nutrient exchange between host and symbiont. As such, arbuscule presence can vary with time according to when nutrient uptake and transfer is demanded by the host plant (Mullen and Schmidt, 1993). Although I observed fescue genotypic differences in occurrence of arbuscules, I measured no tall fescue genotype-specific effects on plant nutrients or biomass in Analysis 1 that might explain the significant arbuscule reduction in CTE45 (Table 4.5). There is some evidence that both host and fungal AMF genotypes may interact to determine presence, abundance, and morphology of different AMF structures such as arbuscules (Demuth et al., 1991; Smith and Smith, 1997). My results suggest that some tall fescue genotypes, such as CTE45, are less inclined to form nutrient-transfer symbioses with AMF than others.
AMF vesicles are thought to function as energy storage organs or as resting spores within or between root cortex cells (Smith and Read, 2008), yet little is known about what host or environmental characteristics specifically control vesicle production (Smith and Smith, 1997). Because endophyte presence reduced vesicle colonization regardless of symbiotic genotype in this study, which varied with my predictions that CTE and NTE effects would differ, the mechanisms producing this effect must be related to characteristics shared by both CTE and NTE strains. Reidinger et al. (2012) found that total concentration of pyrrolizidine alkaloids (senecionine, seneciophylline, jacobine, jacozine and jacoline) was negatively related to AMF vesicle colonization in *Senecio jacobaea*. Because both CTE and NTE endophytes can usually produce loline alkaloids, it is tempting to suggest that presence of loline alkaloids such as N-formylloline, the dominant alkaloid produced by *E. coenophiala* (Bush et al., 1997), may have played a role in the endophyte-related reduction in AMF vesicle abundance observed in this study. However, of the four tall fescue genotypes examined, NTE16 does not produce loline alkaloids while the remaining three genotypes vary significantly in the total concentration of lolines produced [NTE19 > CTE14 > CTE45 (Bourguignon et al., 2015)]. Because the endophyte effect on vesicles was consistent across genotypes, loline alkaloid production was, therefore, likely not the dominant causal factor of the response. In Antunes et al. (2008), presence of CTE+ tall fescue thatch stimulated vesicle production but decreased arbuscule colonization in *Bromus inermis* compared to E− thatch, suggesting that inoculated AMF were stressed by characteristics unique to CTE+ tall fescue, such as the presence of ergot alkaloids. These results contrast with my study, where vesicles decreased in response to endophyte presence regardless of strain. It is likely that
differences in other non-loline alkaloids, or even other non-alkaloid metabolites, shared by both CTE and NTE strains were responsible for decreasing vesicle occurrence.

Some research suggests that AMF vesicles may be formed in response to stressful environmental conditions (Cooke et al., 1993; Smith and Read, 2008). If endophyte presence improved overall plant vigor and reduced plant/microbe stress, then a reduction in vesicle occurrence might be expected. Endophyte presence increased total shoot weight, but only under added precipitation (Fig. 4.7B), and while endophyte effects were observed on root and shoot N:P, shoot P, and root weight (Table 4.6), it is difficult to assess whether these effects indicate improved plant vigor.

Also consistent with my first hypothesis, both tall fescue genotype and endophyte status governed the length of extraradical AMF hyphae in mesic conditions. NTE16 had less ERH than CTE45 and NTE19, but only when endophyte-free. When E+, all symbiotic genotypes had similar ERH levels, which differed from my prediction that NTE symbioses would express intermediate effects compared to CTE. Endophyte presence significantly reduced soil ERH, but only in NTE19 (Fig. 4.3A). These results contrast with prior studies that have reported a negative effect of CTE+ tall fescue on soil AMF either in terms of lipid biomarker abundance (Buyer et al., 2011), or through interfering with AMF colonization of neighboring plants, potentially through effects on soil hyphae (Antunes et al., 2008). The fact that I only found endophyte-symbiosis effects on ERH in NTE19 suggests that production of ergot alkaloids, the predominantly recognized difference between CTE+ and NTE+ tall fescue, was not a causal factor in these results.
Mummey and Rillig (2006) suggest that mechanisms by which plants influence extraradical AMF in soils may include allocation of C resources to AMF, changes in the rate of hyphal decomposition, or changes in active plant biomass present to support extraradical hyphae. However, I did not find consistent tall fescue genotype or endophyte status-mediated effects on plant nutrients or biomass that would help explain the mechanisms creating these ERH results, nor were there any strong correlations between ERH and plant nutrient or biomass characteristics (Pearson r < 0.3 in all cases, Appendix 3). It is possible that the reduction in ERH due to endophyte presence observed in NTE19 (Fig. 4.3A) was due to either to competition between symbionts for C or to changes in the rate of hyphal decomposition, but I cannot explain why this effect was not significant among the other genotypes.

One reason that tall fescue genotype and endophyte presence significantly affected occurrence of specific AMF structures and abundance of ERH may have been differences in AMF species colonizing these plants, as different species exhibit different developmental and functional dynamics of AMF structures such as arbuscules and vesicles (Dodd et al., 2000). In addition, although AMF are often morphologically and functionally distinguished by their production of arbuscules and vesicles, these structures are not necessarily found in all AMF species (Smith and Smith, 1997). I am unable to test whether AMF species differences were driving the trends in my data, but future studies could focus on characterizing how AMF community composition changes due to *E. coenophiala* symbiosis and host-symbiont genetic variability in tall fescue. Overall, my results suggest that both endophyte symbiosis and tall fescue genotype influence AMF investment in different structures, such as arbuscules, vesicles, and extraradical hyphae.
These may lead to divergent long-term responses in ecosystem processes such as nutrient cycling through alterations in presence and functioning of AMF structures such as arbuscules used for nutrient transfer. Although I did not find any effects on soil C or N concentrations in this study, altered abundance of extraradical hyphae in soils could also lead to long term changes in C sequestration (Duchicela et al., 2013; Miller and Jastrow, 1990; Wilson et al., 2009).

4.4.2. Analysis 2: Genotype and endophyte status control belowground symbiont response to climate change

Because I expected physiological differences due to both tall fescue genotype and endophyte status to be sensitive to altered climate conditions (Bourguignon et al., 2015), I hypothesized that the effects of tall fescue symbiotic genotype and endophyte symbiosis on belowground fungi would be altered by climate change factors such as warming and added precipitation. However, this was only partially supported by the results of this study. Vesicle colonization was interactively governed by tall fescue genotype, endophyte status, and warming (Fig 4.5A), and extraradical AMF hyphae in soils were interactively determined by tall fescue genotype, endophyte status, warming, and added precipitation (Fig. 4.6). In contrast, the occurrence of arbuscules was only influenced by endophyte status and precipitation, and tall fescue symbiotic genotype exhibited no main or interactive influence on the rate of root colonization by dark septate endophytes (DSE) or their response to climate change, though DSE were significantly altered by main effects of endophyte status and added heat alone (Table 4.4, Appendix 2A).

Despite being primarily controlled by endophyte status in mesic conditions (Appendix 1A; Analysis 1), the occurrence of vesicles in roots became interactively
governed by fescue and endophyte genotype when subjected to warming. Increased temperature decreased vesicle occurrence in E− clones of NTE19, but stimulated it in E+ clones. However, none of these effects were observed in CTE45 (Fig. 4.5A). Given prior suggestions that vesicle formation may increase due to stressful environmental conditions (Cooke et al., 1993; Smith and Read, 2008), it is possible that warming-induced stress to either the plant or AMF may be causing the observed effects in NTE19. Perhaps higher temperature stimulated competitive interactions between symbionts for plant resources, causing *E. coenophiala* presence to increase AMF vesicles due to nutrient stress, but only in genotype NTE19.

Mack and Rudgers (2008) suggest that *E. coenophiala* possesses both spatial and temporal priority over AMF with respect to plant C. This is because *E. coenophiala* is located in leaf sheaths where C is fixed, compared to AMF in roots, and because *E. coenophiala* is vertically transmitted through seed therefore present in the plant from the beginning, compared to horizontal infection by AMF, which requires time to colonize. Together, these increase the competitive ability of *E. coenophiala* for plant resources compared to belowground symbionts such as AMF. E+ plants generally had greater root and shoot biomass (Fig. 4.10A,B) and thus potentially more plant C resources available for both symbionts, although NTE19 generally had less live, green shoot biomass than CTE45 (Table 4.7). It is possible that endophyte symbiosis significantly alleviated vesicle formation in NTE19 when no heat was added (Fig 4.5A) due to increased plant vigor and abundant C resources, and thus little need for competition between symbionts, similar to the effect observed in Analysis 1. In a prior analysis using the same plants and experimental system as this study, Bourguignon et al. (2015) found that endophyte
symbiosis had no effect on photosynthesis activity under higher temperatures, at least in symbiotic genotype NTE19. Potential warming-related changes in tall fescue physiology for genotype NTE19, though not measured in this study, therefore likely occurred without increases in photosynthetically-produced C. Under higher temperatures, spatial and temporal priority of *E. coenophiala* may have competitively resulted in fewer plant resources allocated to AMF, thus inducing higher vesicle formation in E+ clones of NTE19 (Fig 4.5A).

Consistent with my hypothesis, and in addition to the genotype- and endophyte-controls on ERH found in mesic conditions with Analysis 1, both of these factors interacted with additional heat and precipitation in Analysis 2 (Fig. 4.6). Although, the outcome of this interaction differed slightly from my prediction that warming may stimulate extraradical AMF hyphae in soil. Compared to ambient climate conditions, warming and added precipitation together significantly increased the length of soil extraradical hyphae in E− clones of NTE19, and in E+ clones of CTE45 (Fig. 4.6). These results are partially supported by prior studies that found increased length of soil extraradical AMF hyphae in response to warming (Bunn et al., 2009; Heinemeyer and Fitter, 2004; Rillig et al., 2002). However, I observed a stimulatory warming effect only in combination with added precipitation in NTE19, albeit only significantly among E− clones, and only in E+ clones of CTE45. Novas et al. (2011) previously demonstrated that exudates of *Bromus setifolius* infected with an asexual *Epichloë* endophyte increased AMF hyphal branching and length compared to exudates of uninfected plants, and that this effect varied by endophyte strain. This may explain why I observed genotype-specific effects of endophyte presence on extraradical AMF hyphal response to climate
change in this study. These results suggest that although endophyte symbiosis alone may not significantly impact extraradical AMF hyphae, it can alter soil AMF response to future climate change in ways that are unique to different E. coenophiala-tall fescue associations.

Physical and chemical activities of AMF hyphal networks in soil are substantial contributors to improved soil physical structure and C sequestration [e.g., (Duchicela et al., 2013; Miller and Jastrow, 1990; Wilson et al., 2009)]. Although I observed a stimulatory effect of warming and endophyte status on soil N concentrations, this difference was very small (under warming, −0.02% in E− vs. E+ samples; Fig. 4.13) and I did not find any effects on soil C concentrations after two years of plant growth. Despite the lack of short-term change, under future climatic conditions, differential responses between host-symbiont genotypes and presence of E. coenophiala in tall fescue could cause these ecosystem properties to diverge with time in pastures containing different grass-endophyte associations.

Unlike the primarily genotypic control of AMF arbuscule colonization found in Analysis 1, I found a significant endophyte-related decrease in the rate of AMF arbuscule presence that was ameliorated by added growing season precipitation in Analysis 2 (Fig. 4.4). Regardless of precipitation level, E+ plants generally exhibited greater root and total shoot biomass than E− tall fescue (Fig. 4.10A,B), which means there should potentially have been enough plant resources, such as photosynthetically-produced C, for both symbionts. Because arbuscules are the AMF structures responsible for host-symbiont resource exchange (Smith and Smith, 1989), this suggests that competitive interactions between E. coenophiala and AMF caused a shift in AMF’s functional role within the
plant away from nutritional symbiosis. However, I note that the endophyte effect was only significant in Analysis 2, where I was able to include only two tall fescue genotypes, but not in Analysis 1 (Appendix 1A), which included all four tall fescue genotypes. This potentially highlights the importance of examining multi-symbiont relationships within a variety of host genotypes, as interactions may become more or less apparent depending on plant genetics.

Unlike the AMF structures discussed above, the rate of tall fescue root colonization by dark septate endophytes (DSE) was not significantly influenced by tall fescue genetics in this study, which did not support my hypotheses. Instead, endophyte status and higher temperatures individually governed the rate of tall fescue root colonization by DSE. Across all climate treatments, presence of *E. coenophiala* significantly decreased DSE colonization (Table 4.4). These results are in contrast to a recent study by Vandegrift et al. (2015), who found that coinfection of *Agrostis capillaris* with an aboveground asexual *Epichloë* endophyte did not significantly impact belowground colonization by DSE. Symbiosis with *Epichloë* canceled a negative effect of DSE on plant biomass in Vandegrift et al. (2015), which I also glimpsed in my study through a weak negative correlation between DSE and plant weight characteristics (Appendix 3). Yet, Vandegrift et al. (2015) were unable to investigate differences in either host or symbiont genotypes. I found significant and consistent endophyte effects on DSE for the two tall fescue genotypes used in Analysis 2 (Table 4.4, Appendix 2A), but this effect disappeared when all four genotypes were analyzed under only mesic conditions (Appendix 1A). This, like the endophyte effect on arbuscules above,
highlights the importance of examining multi-symbiont interactions within multiple host genotypes and in multiple climates.

DSE were originally described as “pseudomycorrhiza” because they exhibit traits similar to both endo- and ectomycorrhiza associations, such as residing intracellularly within plant roots yet forming melanized hyphae and microsclerotia that are distinct from AMF (Melin, 1922). They are increasingly reported to colonize plants roots across various ecosystems, but are less-studied than AMF, and much remains to be determined about their functional roles within plants (Mandyam and Jumpponen, 2014). Thus far, literature results suggest that DSE express a continuum of interactions from parasitic to mutualistic similar to that of AMF, and both host and fungal genotype are important in determining host response to DSE (Mandyam and Jumpponen, 2014). When DSE express mycorrhiza-like functions, they appear to contribute to N uptake and use efficiency (Alberton et al., 2009; Newsham, 2011), though this is not always the case depending on environmental conditions such as organic C and N availability (Mayerhofer et al., 2013).

In my study, significant reduction of DSE colonization due to endophyte symbiosis shows that *E. coenophiala* and DSE directly interacted within host plants (Table 4.4). Root N concentration exhibited a weak positive correlation with DSE colonization (Appendix 3; Regression p < 0.0001, $R^2 = 0.18$), potentially suggesting that DSE were somewhat important for N uptake in tall fescue. In Vandegrift et al. (2015), they proposed that potential N contributions by DSE was not enough to offset the fitness cost incurred by allocating plant C to DSE. Yet, when *Epichloë* was also present, N contributed by DSE may have been used for protective alkaloid biosynthesis by *Epichloë*, potentially offsetting the fitness cost of maintaining both symbionts in addition to AMF.
(Vandegrift et al., 2015). Significant endophyte-related reductions in DSE in my study suggest that, as proposed in Vandegrift et al. (2015), potential benefits to N uptake may not have been enough to offset the plant C costs of maintaining both symbionts.

It is interesting that, in terms of plant resource allocation and interactions between multiple symbionts, DSE were the only belowground fungi directly and consistently inhibited by aboveground *E. coenophiala* status in this study, not AMF (Appendix 2A). Little is known about the evolutionary and historical linkages between plants and DSE, but AMF are known to have associated with plants over 450 million years ago (Redecker et al., 2000), associate with nearly 80% of terrestrial plants (Smith and Read, 2008), and form evolutionarily persistent, widespread symbioses with many plant species (Selosse and Le Tacon, 1998). The evidence discussed above illustrates that AMF structures and potentially functional roles within the plant were altered due to both endophyte symbiosis and tall fescue genetics, suggesting that the nature and function of AMF symbioses at this site are intimately controlled by plant genetics. Despite the perception that these are important, genetically-controlled belowground symbionts known for mutualistic nutrient transfer (Demuth et al., 1991; Smith and Smith, 1997), AMF colonization was not strongly linked to any plant nutrient or biomass characteristics in this study (Appendix 3).

While little is known about non-nutritional roles of DSE within host plants, studies suggest that AMF can provide additional services to hosts, such as alleviating environmental stress (Dashtebani et al., 2014; Hajiboland, 2013), protection from microbial pathogens (Abhiniti et al., 2013; Goicoechea et al., 2010), and even transportation of plant allelochemicals through soil mycelia (Achatz et al., 2014). In this study, I am unable to determine whether AMF served additional non-nutrient roles, yet
future studies could investigate these possibilities. In addition, unlike ‘pseudomycorrhizal’ DSE, for whom there is very limited evidence for formation of mycelial networks (Jumpponen, 1999), the presence of a connected mycorrhizal network through AMF for potential transfer of nutrients or other non-nutritional services between plants (Bever et al., 2010; Simard and Durall, 2004) may also make AMF a more valuable symbiosis to host plants. It is possible that, because the addition of aboveground *E. coenophiala* symbiosis competitively inhibited DSE but not AMF, AMF exhibited a stronger symbiosis with plants than DSE.

Independent of the antagonistic effects of aboveground *E. coenophiala*, warming doubled the rate of root DSE colonization in both symbiotic genotypes (Table 4.4, Appendix 2A). These results support findings in Olsrud et al. (2010), where experimental warming also increased plant root colonization by DSE. However, Vandegrift et al. (2015) found that soil warming negatively affected DSE colonization, as did increased soil water availability. It is possible that the stimulatory effect of heat on DSE colonization in my study is related to warming-induced reductions in shoot N, root C, and root P, or increases in shoot and root N:P and root C:N (Table 4.9, Appendix 2B,C). I note that none of these nutrient characteristics were clearly correlated with DSE colonization (Pearson r < 0.3 in all cases, Appendix 3), except for a weak positive association between DSE colonization and root N:P (Regression p < 0.0001, R² = 0.23). I cannot determine, however, whether warming directly increased DSE colonization and thereby produced similar increases in root N:P as a result of potentially increased N-uptake through DSE, or whether warming-induced reduction of root P, but not root N, simply influenced the ratio of N to P. Because the ecosystem effects of DSE are not well-
studied (Mandyam and Jumpponen, 2014), I cannot confidently predict how increased DSE colonization due to climate warming will impact future plant and soil communities. The warming-induced increase in DSE colonization observed in this study may portend future shifts in belowground fungal community structure due to climate warming, yet further research is needed to elucidate their functional role and potential ecosystem effects.

4.5. Conclusions

In this study, I found that although tall fescue symbiotic genotype and aboveground *E. coenophiala* symbiosis did not significantly alter the total rate of colonization by belowground AMF, they did affect the abundance of specific AMF structures such as arbuscules in roots and extraradical hyphae in soils. Tall fescue genotypes differed in their inclination to form nutritional symbioses with AMF, while *E. coenophiala* presence appeared to indirectly alleviate AMF stress, indicated by decreased vesicle production, potentially through stimulatory effects on tall fescue biomass or resource availability. Endophyte symbiosis significantly decreased the length of extraradical AMF hyphae in mesic conditions, perhaps through reduction in plant C allocated to AMF, but only in one tall fescue genotype. Genotypic and endophyte symbiosis controls on belowground fungi were altered after two years subject to predicted climate change conditions of added heat and growing season precipitation. For example, where plant biomass increases due to endophyte symbiosis had reduced stress-induced AMF vesicles in mesic conditions, higher temperatures changed this to a more antagonistic relationship in one tall fescue genotype, indicated by increased vesicle production due to endophyte symbiosis. Warming and added precipitation together
stimulated extraradical AMF hyphae compared to ambient conditions, yet this was significant in E− individuals of one genotype and E+ individuals of another.

Contrary to my hypotheses that plant and endophyte genetics would influence belowground fungi either in ambient or altered climate conditions, root colonization by dark septate endophytes was instead independently regulated by presence of aboveground E. coenophiala and climate warming. Symbiosis decreased DSE colonization irrespective of plant or endophyte genotype or climate treatment, despite warming doubling the rate of DSE colonization. Despite multiple main or interactive effects of tall fescue symbiotic genotype, endophyte status, and climate change factors on plant nutrient and biomass characteristics after two years, none of these alterations appeared to either follow or be responsible for changes in belowground fungi.

Although different associations between tall fescue and E. coenophiala may not alter the total rate of AMF colonization, I have demonstrated that both host-symbiont genetic variation and E. coenophiala symbiosis in tall fescue result in different plant and endophyte interactions with AMF, resulting in different AMF structures and potentially altering the functional role of these belowground symbionts. These genotype and endophyte controls on belowground fungi are altered under predicted climate change conditions such as warming and added precipitation, which may cause grasslands hosting different E. coenophiala-tall fescue associations to diverge in terms of belowground fungal communities and nutrient characteristics such as C sequestration. Because genetically varied associations between E. coenophiala and tall fescue are increasingly widespread in grassland ecosystems, these findings will be important for predicting long
term response of these ecosystems, such as changes in above- and belowground communities and nutrient cycling, to future climate change.
4.6. Tables and Figures

Table 4.1 Presence (+) or absence (−) of fungal genes, representing either fungal mating types or alkaloid biosynthesis loci, detected in tall fescue tissue. These were used to distinguish between genetic profiles of *E. coenophiala* associated with E+ clones of each tall fescue genotype in this study. Modified from Table 2 in Takach and Young (2014).

<table>
<thead>
<tr>
<th>Target loci</th>
<th>CTE14, <em>E. coenophiala</em> profile 1</th>
<th>CTE45, <em>E. coenophiala</em> profile 2</th>
<th>NTE16, FaTG-4</th>
<th>NTE19, <em>E. coenophiala</em> profile 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>tefA</em>†</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mating Type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>mtAC</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>mtBA</em></td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Ergot Alkaloids (EAS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>dmaW</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td><em>lpsB</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<tr>
<td>Lolines (LOL)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>lolC</em></td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td><em>lolA</em></td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Peramine</td>
<td></td>
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<tr>
<td><em>perA-R</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>perA-T2</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Indole-diterpenes (IDT/LTM)</td>
<td></td>
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<tr>
<td><em>idtG</em></td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>idtQ</em></td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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</table>

†Highly conserved fungal gene denoting endophyte presence in tall fescue tissue.
Table 4.2 Number of replicates available for statistical analyses within each climate treatment, tall fescue symbiotic genotype, and endophyte status.

<table>
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<tbody>
<tr>
<td>CTE 14</td>
<td>5</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>3</td>
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</tr>
<tr>
<td>CTE 45</td>
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<td>4</td>
<td>5</td>
<td>4</td>
<td>3</td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>NTE 16</td>
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<td>3</td>
<td>4</td>
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</tr>
<tr>
<td>NTE 19</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>4</td>
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</tbody>
</table>

Table 4.3 Main effect of tall fescue symbiotic genotype (Analysis 1, TFtype p = 0.0053) on the rate of arbuscule presence in tall fescue roots. Values are means ± 1 S.E., and values with different letters are significantly different (α < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Arbuscules (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTE14</td>
<td>20 (3)</td>
</tr>
<tr>
<td>CTE45</td>
<td>9 (2)</td>
</tr>
<tr>
<td>NTE16</td>
<td>16 (2)</td>
</tr>
<tr>
<td>NTE19</td>
<td>13 (2)</td>
</tr>
</tbody>
</table>

Table 4.4 Main effects of heat (0 or + Heat) and endophyte status (E− or E+; Analysis 2) on the rate of dark septate endophyte (DSE) colonization (%) in tall fescue roots. Values are means ± 1 S.E., and are significantly different across rows (p-value shown for main effect).

<table>
<thead>
<tr>
<th>Treatment Level</th>
<th>0/−</th>
<th>+</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat</td>
<td>10.00 (1.51)</td>
<td>21.15 (3.10)</td>
<td>0.0193</td>
</tr>
<tr>
<td>Endophyte Status</td>
<td>18.27 (2.78)</td>
<td>12.08 (2.12)</td>
<td>0.0318</td>
</tr>
</tbody>
</table>
Table 4.5 Main effect of tall fescue symbiotic genotype (Analysis 1) on plant nutrient characteristics in tall fescue tissue. Values are means ± 1 S.E. Across rows, means with different letters are significantly different (α = 0.05, p-value shown for main effect).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CTE 14</th>
<th>CTE 45</th>
<th>NTE 16</th>
<th>NTE 19</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot N (%)</td>
<td>0.97 (0.03)\textsuperscript{b}</td>
<td>0.98 (0.04)\textsuperscript{b}</td>
<td>1.10 (0.04)\textsuperscript{a}</td>
<td>1.19 (0.04)\textsuperscript{a}</td>
<td>0.0001</td>
</tr>
<tr>
<td>Shoot N:P</td>
<td>3.43 (0.13)\textsuperscript{b}</td>
<td>3.52 (0.13)\textsuperscript{b}</td>
<td>4.10 (0.20)\textsuperscript{a}</td>
<td>4.15 (0.19)\textsuperscript{a}</td>
<td>0.0074</td>
</tr>
<tr>
<td>Shoot C:N</td>
<td>40.66 (1.26)\textsuperscript{a}</td>
<td>36.47 (1.10)\textsuperscript{b}</td>
<td>34.32 (1.14)\textsuperscript{b}</td>
<td>30.26 (0.87)\textsuperscript{c}</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 4.6 Main effect of endophyte status (Analysis 1) on nutrient characteristics and root biomass of tall fescue tissue. Values are means ± 1 S.E., and p-values are shown for the significant differences across rows.

<table>
<thead>
<tr>
<th>Endophyte Status</th>
<th>–</th>
<th>+</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot P (%)</td>
<td>0.22 (0.01)</td>
<td>0.24 (0.01)</td>
<td>0.0013</td>
</tr>
<tr>
<td>Shoot N:P</td>
<td>4.08 (0.13)</td>
<td>3.55 (0.11)</td>
<td>0.0031</td>
</tr>
<tr>
<td>Root N:P</td>
<td>4.80 (0.27)</td>
<td>3.96 (0.18)</td>
<td>0.0300</td>
</tr>
<tr>
<td>Root weight (g)</td>
<td>1.90 (0.28)</td>
<td>2.95 (0.31)</td>
<td>0.0129</td>
</tr>
</tbody>
</table>
Table 4.7 Main effect of tall fescue symbiotic genotype (Analysis 2) on nutrient characteristics and green shoot biomass of tall fescue tissue. Values are means ± 1 S.E., and p-values are shown for the significant differences across rows.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CTE45</th>
<th>NTE19</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot N (%)</td>
<td>1.06 (0.04)</td>
<td>1.24 (0.04)</td>
<td>0.0009</td>
</tr>
<tr>
<td>Root P (%)</td>
<td>0.142 (0.004)</td>
<td>0.156 (0.004)</td>
<td>0.0099</td>
</tr>
<tr>
<td>Root C:N</td>
<td>39.95 (1.13)</td>
<td>32.29 (0.89)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Green shoot weight (g)</td>
<td>2.70 (0.40)</td>
<td>1.82 (0.36)</td>
<td>0.0187</td>
</tr>
</tbody>
</table>

Table 4.8 Main effect of endophyte status (Analysis 2) on green and dead shoot biomass of tall fescue tissue. Values are means ± 1 S.E., and p-values are shown for the significant differences across rows.

<table>
<thead>
<tr>
<th>Endophyte Status</th>
<th>−</th>
<th>+</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green shoot weight (g)</td>
<td>1.51 (0.26)</td>
<td>2.90 (0.43)</td>
<td>0.0111</td>
</tr>
<tr>
<td>Dead shoot weight (g)</td>
<td>1.18 (0.18)</td>
<td>1.74 (0.19)</td>
<td>0.0299</td>
</tr>
</tbody>
</table>

Table 4.9 Main effect of heat (Analysis 2) on nutrient characteristics of tall fescue tissue. Values are means ± 1 S.E., and p-values are shown for the significant differences across rows.

<table>
<thead>
<tr>
<th>Heat</th>
<th>−</th>
<th>+</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot N (%)</td>
<td>1.23 (0.05)</td>
<td>1.08 (0.03)</td>
<td>0.0368</td>
</tr>
<tr>
<td>Shoot N:P</td>
<td>3.83 (0.12)</td>
<td>4.76 (0.21)</td>
<td>0.0005</td>
</tr>
<tr>
<td>Root P (%)</td>
<td>0.157 (0.004)</td>
<td>0.138 (0.004)</td>
<td>0.0090</td>
</tr>
<tr>
<td>Root N:P</td>
<td>4.39 (0.22)</td>
<td>6.72 (0.36)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Root C (%)</td>
<td>29.98 (0.91)</td>
<td>35.43 (0.75)</td>
<td>0.0009</td>
</tr>
<tr>
<td>Root C:N</td>
<td>38.17 (1.10)</td>
<td>33.71 (1.28)</td>
<td>0.0284</td>
</tr>
</tbody>
</table>
Figure 4.1 Field design of UK Forage Climate Change project established in 2008 at the UK Spindletop research farm in Lexington, KY. Factorial combinations of added heat (H), and added precipitation (P) were applied to hexagonal, 5.8 m² plots (C = control/ambient climate conditions). Cloned pairs of four tall fescue genotypes, where one clone was endophyte-infected (E+) and one clone was endophyte-free (E−) were transplanted into each climate treatment plot in 2011 (n = 8 plants per plot). E+ clones of tall fescue genotypes 14 and 45 contained common toxic endophyte (CTE) strains, whereas E+ clones of tall fescue genotypes 16 and 19 contained non-toxic endophyte (NTE) strains.
Figure 4.2 Monthly averages of A) soil volumetric moisture content, B) soil temperature, and C) air temperature measured within each climate treatment. Values are means ± 1 S.E. for each month during the two-year period between clone pair planting in late October 2011, and harvest in October 2013.
Figure 4.3 Interactive effects of tall fescue symbiotic genotype and endophyte status from the Control and +Precip treatments only (Analysis 1) on A) Length of extraradical AMF hyphae in soil associated with tall fescue plants, B) the ratio of C:N in belowground tall fescue root tissue, and C) weight of dead tissue from aboveground tall fescue shoots. Bars indicate means ± 1 S.E. Within each panel, bars sharing no common letter (a, b, c) indicate significant differences between means (α = 0.05).
Figure 4.4 Interactive effects of endophyte status and added precipitation on rate of AMF arbuscule colonization in tall fescue roots of the CTE45 and NTE19 symbiotic genotypes only (Analysis 2). Bars indicate means ± 1 S.E., and those sharing no common letter (a, b) indicate significant differences between means (α = 0.05).
Figure 4.5 Interactive effect of tall fescue symbiotic genotype, endophyte status, and added heat (Analysis 2) on A) rate of AMF vesicle colonization in tall fescue roots and B) C:N ratio of tall fescue shoot tissue. Bars indicate means ± 1 S.E. Within each panel, bars sharing no common letter (a, b) indicate significant differences between means (α = 0.05). All sample values were 0 within NTE19, E−, +Heat (marked with an asterisk*; n = 5). For ease of interpretation, significant differences are shown only within each symbiotic genotype group. NS indicates no significant differences within a group.
Figure 4.6 Interactive effects of tall fescue symbiotic genotype, endophyte status, added heat, and added precipitation (Analysis 2) on extraradical AMF hyphae in root-associated soils. Bars indicate means ± 1 S.E. Within each panel, bars sharing no common letter (a, b) indicate significant differences between means (α = 0.05). For ease of interpretation, significant differences are only shown between climate treatments within endophyte status x tall fescue symbiotic genotype groups. NS indicates no significant differences within a group, while bar marked with an asterisk (*) signifies low replication (n = 1).
Figure 4.7 Interactive effects of endophyte status and added precipitation (Analysis 1) on A) dead shoot weight and B) total shoot weight in tall fescue. Bars indicate means ± 1 S.E. Within each panel, bars sharing no common letter (a, b) indicate significant differences between means (α = 0.05).
**Figure 4.8** Interactive effects of tall fescue symbiotic genotype, endophyte status, and added precipitation on the weight of green tissue in aboveground tall fescue shoots (Analysis 1). Bars indicate means ± 1 S.E., and those sharing no common letter (a, b) indicate significant differences between means within a symbiotic genotype (α = 0.05). NS indicates no significant differences within a group.
Figure 4.9 Interactive effects of tall fescue symbiotic genotype and added heat (Analysis 2) on A) shoot P (%) and B) root N (%) in tall fescue. Bars indicate means ± 1 S.E.; within each panel, bars sharing no common letter (a, b) indicate significant differences between means (α = 0.05).
Figure 4.10 Interactive effects of tall fescue symbiotic genotype and endophyte status on A) root weight and B) total shoot weight of tall fescue (Analysis 2). Bars indicate means ± 1 S.E. Within each panel, bars sharing no common letter (a, b) indicate significant differences between means (α = 0.05).
Figure 4.11 Interactive effects of added heat and precipitation (Analysis 2) on A) shoot C:N ratio, B) green shoot weight and C) total shoot weight of tall fescue. Bars indicate means ± 1 S.E. Within each panel, bars sharing no common letter (a, b) indicate significant differences between means ($\alpha = 0.05$).
Figure 4.12 Interactive effects of tall fescue symbiotic genotype, endophyte status, and added precipitation (Analysis 2) on tall fescue A) shoot P (%), B) shoot N:P ratio, and C) shoot C:N ratio. Bars indicate means ± 1 S.E. Within each panel, bars sharing no common letter (a, b) indicate significant differences between means (α = 0.05). A,B) For ease of interpretation, significant differences are shown only within each symbiotic genotype group. NS indicates no significant differences within a group. C) No significant differences existed within tall fescue symbiotic genotype groups. Instead, significant differences are shown within endophyte status groups.
Figure 4.13 Interactive effects of endophyte status and added heat on N concentration (%) of soils associated with tall fescue roots. Bars indicate means ± 1 S.E.
Chapter Five

Research Synthesis and Conclusions

In this dissertation, I demonstrated that manipulating *E. coenophiala*-tall fescue associations, both in terms of endophyte presence and host-symbiont genetic combinations, altered certain plant-microbe-soil interactions in temperate pasture ecosystems. For example, I found that *E. coenophiala* presence and strain affected tall fescue’s inclination to utilize nitrogen produced from biological transformations of \(\text{N}_2\), such as that provided by legume symbiosis. I also discovered that *E. coenophiala* presence and strain may not significantly alter the rate of colonization by concomitant belowground fungal symbionts such as AMF and DSE within a single cultivar, especially in mesic, temperate, high P environments. Yet, I also found that genotypic variation in tall fescue in addition to endophyte presence and strain does alter the functional capacity of belowground AMF, such as altering AMF investment in nutrient transfer organs (arbuscules), stress-induced energy storage structures (vesicles), and extraradical hyphae in soils. Further, I found that these genotypic and endophyte controls on belowground AMF are altered under future climate change conditions such as warming and increased precipitation, which can either ameliorate or exacerbate competitive interactions between these symbionts.

The results of this dissertation suggest that different *E. coenophiala*-tall fescue associations, through alteration of plant-microbe-soil interactions, may differentially affect pasture ecosystem structure and function. For example, in Chapter 2, AR542 NTE+ tall fescue incorporated significantly less biologically-fixed N into aboveground plant tissue than both CTE+ and AR584 NTE+ tall fescue when grown immediately next to red
clover. This suggests that certain *E. coenophiala* and host-symbiont genetic combinations are better suited to pasture ecosystems designed to maximize biological cycling of nutrients such as through legume symbioses. Yet, both CTE+ and NTE+ tall fescue incorporated significantly less biologically-fixed N into aboveground plant tissue than E− tall fescue when plants were spatially distant (> 45 cm) from red clover. This suggests that absence of *E. coenophiala* symbiosis in tall fescue allowed greater support for transfer of biologically-fixed N within plots, potentially through impacts on belowground community characteristics such as mycorrhizal networks.

Selection and deployment of genetically different *E. coenophiala*-tall fescue associations will also alter functional interactions between concomitant symbionts such as AMF, which will impact ecosystem properties such as nutrient cycling and C sequestration. For example, in Chapter 3 I did not find endophyte presence- or strain-related differences in belowground AMF or DSE colonization within one cultivar of tall fescue, but in Chapter 4 I discovered that tall fescue and *E. coenophiala* genotype and endophyte status altered the presence of functionally different AMF structures such as arbuscules, vesicles, and ERH.

Such interactions will be further altered by future climate change conditions, potentially impacting composition and function of belowground communities and associated ecosystem processes such as nutrient cycling. For example, *E. coenophiala* presence stimulated stress-induced AMF vesicle colonization in NTE19 under climate warming, but had reduced vesicle presence across all four genotypes in mesic conditions, which may suggest that aboveground endophytes became more antagonistic towards AMF under conditions of elevated heat, but only in this genotype. Although *E.
coenophiala decreased extraradical AMF hyphae of only genotype NTE19 in mesic conditions, warming and added precipitation together stimulated extraradical AMF hyphae compared to ambient conditions in E− individuals of NTE19 and E+ individuals of CTE45. Although I did not find any short-term (two year) effects on soil C, pastures supporting different tall fescue genotypes and E. coenophiala-tall fescue associations may diverge in long-term ERH-related ecosystem properties such as soil C sequestration under future climate conditions. Interestingly, the rate of root colonization by dark septate endophytes (DSE) was not controlled by host genetics in this study, in contrast to effects on AMF. Instead, aboveground E. coenophiala symbiosis reduced DSE within CTE45 and NTE19, suggesting that DSE colonization is determined by either plant C budgets, priority effects of aboveground symbionts, or other common endophyte signaling effects, rather than genetics. In addition, warming doubled DSE colonization, suggesting that belowground fungal communities may be altered under predicted climate change.

The results of this research highlight a number of questions for future research. For example, AMF investment in structure such as arbuscules, vesicles, and extraradical hyphae differ between species (Dodd et al., 2000; Hempel et al., 2007), and some species may not express one or more of these structures in detectable amounts (Smith and Smith, 1997), which may explain some of the results from Chapter 3 and 4. Future studies of epichloïd endophyte effects on belowground symbioses both within host plants and in associated soil should therefore investigate differences in fungal species. Because I found altered amounts of functionally-different AMF structures in Chapter 4, I also propose that future research efforts mechanistically explore how functional roles of multiple symbionts within plants can change regarding services other than plant growth and
nutrient uptake, such as pathogen deterrence, and how these interactions will govern plant-microbe-soil response to future climate change. In addition, each of these studies took place in a relatively unstressed mesic, high P, temperate ecosystem. The outcomes of this research would very likely differ among other soils or field conditions, and should be mechanistically explored in future studies.

Finally, this dissertation focuses solely on the specific association between *E. coenophiala* and tall fescue, which has great importance in U.S. pasture ecosystems. Yet, related but diverse *Epichloë*-grass species combinations are found in agronomic and natural ecosystems worldwide, in both native and exotic grasses (Semmartin et al., 2015). Evidence exists that host-symbiont interactions, and interactions with plant communities and other organisms, differ between ecosystems in which the grass-endophyte symbiosis is native and primarily agronomic ecosystems to which it is introduced (Saikkonen et al., 1998; Saikkonen et al., 2006). I advocate for the *E. coenophiala*-tall fescue symbiosis as a convenient and valuable model system, especially because of its agronomic implications. However, we will never gain a complete understanding of the complex relationships between plants, microbes, and soils without considering these effects in other *Epichloë*-grass systems. In future studies, we must extend the use of *Epichloë*-grass models in studying plant-microbe-soil interactions to a greater variety of host-symbiont species associations, and also investigate these relationships across multiple ecosystems spanning a range of biotic and abiotic conditions.
### Appendix 1A

**ANOVA results for the effects of independent treatment variables considered in Analysis 1 on fungal parameters.** Significant p-values ($\alpha = 0.05$) are denoted with red bold type.

<table>
<thead>
<tr>
<th>Response Variable</th>
<th>ERH</th>
<th>Total AMF</th>
<th>AMF Arbuscules</th>
<th>AMF Vesicles</th>
<th>AMF Hyphae</th>
<th>DSE</th>
<th>soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precip</td>
<td>3.20 1.7 0.59 0.3085</td>
<td>3.20 2.5 0.48 0.3980</td>
<td>3.20 0.9 0.27 0.4480</td>
<td>3.20 0.3 0.13 0.4480</td>
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<td>3.20 1.7 0.59 0.3085</td>
<td>3.20 1.7 0.59 0.3085</td>
</tr>
<tr>
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<td>1.20 1.2 0.40 0.2700</td>
<td>1.20 0.8 0.45 0.4480</td>
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<td>1.20 1.2 0.40 0.2700</td>
<td>1.20 1.2 0.40 0.2700</td>
<td>1.20 1.2 0.40 0.2700</td>
</tr>
<tr>
<td>TFtype x Precip</td>
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<td>3.20 1.1 0.37 0.4480</td>
<td>3.20 0.9 0.39 0.4480</td>
<td>3.20 1.1 0.37 0.4480</td>
<td>3.20 1.1 0.37 0.4480</td>
<td>3.20 1.1 0.37 0.4480</td>
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</tr>
<tr>
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<td>1.20 0.5 0.28 0.4511</td>
<td>1.20 0.5 0.28 0.4511</td>
<td>1.20 0.5 0.28 0.4511</td>
</tr>
<tr>
<td>Estatus x Precip</td>
<td>3.20 0.9 0.39 0.4480</td>
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<td>3.20 0.8 0.45 0.4511</td>
<td>3.20 0.8 0.45 0.4511</td>
<td>3.20 0.8 0.45 0.4511</td>
</tr>
<tr>
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<td>3.20 0.3 0.13 0.4511</td>
<td>3.20 1.1 0.37 0.4511</td>
<td>3.20 1.1 0.37 0.4511</td>
<td>3.20 1.1 0.37 0.4511</td>
<td>3.20 1.1 0.37 0.4511</td>
</tr>
<tr>
<td>TFtype x Estatus x Precip</td>
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<td>3.20 1.0 0.36 0.4480</td>
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<td>3.20 1.0 0.36 0.4480</td>
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<td>3.20 1.0 0.36 0.4480</td>
<td>3.20 1.0 0.36 0.4480</td>
</tr>
</tbody>
</table>

† Numerator, denominator degrees of freedom
Appendix 1B.

ANOVA results for the effects of independent treatment variables considered in Analysis 1 on shoot nutrient parameters. Significant p-values ($\alpha = 0.05$) are denoted with red bold type.

<table>
<thead>
<tr>
<th>Response Variable</th>
<th>Independent Variable</th>
<th>df</th>
<th>F</th>
<th>p</th>
<th>df</th>
<th>F</th>
<th>p</th>
<th>df</th>
<th>F</th>
<th>p</th>
<th>df</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot C:N</td>
<td>Precip</td>
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<td>0.05</td>
<td>0.8385</td>
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<td>1,4</td>
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<td>Shoot N:TFtype</td>
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<td>0.3742</td>
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† Numerator, denominator degrees of freedom.
Appendix 1C.
ANOVA results for the effects of independent treatment variables considered in Analysis 1 on root nutrient parameters. Significant p-values ($\alpha = 0.05$) are denoted with red bold type.
Appendix 1D. ANOVA results for the effects of independent treatment variables considered in Analysis 1 on tall fescue biomass and parameters and on soil C and N concentrations. Significant p-values ($\alpha = 0.05$) are denoted with red bold type.

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<th>Soil N</th>
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<th>Root Weight</th>
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† Numerator, denominator degrees of freedom.
Appendix 2A. ANOVA results for the effects of independent treatment variables considered in Analysis 2 on fungal parameters. Significant p-values (\(\alpha = 0.05\)) are denoted with bold type.

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<th>AMF Hyphae</th>
<th>AMF Vesicles</th>
<th>AMF Arbuscules</th>
<th>Total AMF</th>
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<td>df†</td>
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<td>p</td>
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†Numerator, denominator degrees of freedom
‡Only genotypes CTE45 and NTE19 considered in Analysis 2
Appendix 2B.

ANOVA results for the effects of independent treatment variables considered in Analysis 2 on shoot nutrient parameters. Significant p-values ($\alpha = 0.05$) are denoted with red bold type.

<table>
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<th>p</th>
<th>df†</th>
<th>F</th>
<th>p</th>
<th>df†</th>
<th>F</th>
<th>p</th>
<th>df†</th>
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<th>F</th>
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</table>

†Numerator, denominator degrees of freedom
‡Only genotypes CTE45 and NTE19 considered in Analysis 2
Appendix 2C. ANOVA results for the effects of independent treatment variables considered in Analysis 2 on root nutrient parameters. Significant p-values ($\alpha = 0.05$) are denoted with red bold type.

<table>
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<th>p</th>
<th>df†</th>
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<th>p</th>
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<td>0.5374</td>
<td>1,15</td>
<td>0.04</td>
<td>0.8423</td>
<td>1,15</td>
<td>0.16</td>
<td>0.6927</td>
<td>1,15</td>
<td>0.86</td>
<td>0.3691</td>
</tr>
<tr>
<td>TFtype x Estatus</td>
<td>1,15</td>
<td>0.65</td>
<td>0.4335</td>
<td>1,15</td>
<td>3.40</td>
<td>0.0852</td>
<td>1,15</td>
<td>0.2</td>
<td>0.6597</td>
<td>1,15</td>
<td>0.20</td>
<td>0.6613</td>
<td>1,15</td>
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<td>0.4461</td>
</tr>
<tr>
<td>TFtype x Estatus x Heat</td>
<td>1,15</td>
<td>0.02</td>
<td>0.9009</td>
<td>1,15</td>
<td>4.34</td>
<td>0.0547</td>
<td>1,15</td>
<td>0.92</td>
<td>0.3530</td>
<td>1,15</td>
<td>1.48</td>
<td>0.2423</td>
<td>1,15</td>
<td>0.18</td>
<td>0.6792</td>
</tr>
<tr>
<td>TFtype x Estatus x Precip</td>
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<td>1.92</td>
<td>0.1859</td>
<td>1,15</td>
<td>0.00</td>
<td>0.9902</td>
<td>1,15</td>
<td>0.00</td>
<td>0.9902</td>
<td>1,15</td>
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<tr>
<td>TFtype x Estatus x Heat x Precip</td>
<td>1,15</td>
<td>0.36</td>
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<td>0.9395</td>
<td>1,15</td>
<td>0.14</td>
<td>0.7106</td>
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<td>0.18</td>
<td>0.6792</td>
<td>1,15</td>
<td>0.28</td>
<td>0.6058</td>
</tr>
</tbody>
</table>

†Numerator, denominator degrees of freedom
‡Only genotypes CTE45 and NTE19 considered in Analysis 2
Appendix 2D. ANOVA results for the effects of independent treatment variables considered in Analysis 2 on tall fescue biomass and parameters and on soil C and N concentrations. Significant p-values (\( \alpha = 0.05 \)) are denoted with red bold type.

<table>
<thead>
<tr>
<th>Response Variable</th>
<th>Root weight</th>
<th>Total shoot weight</th>
<th>Green shoot weight</th>
<th>Dead shoot weight</th>
<th>Soil C</th>
<th>Soil N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Independent Variable</td>
<td>df†</td>
<td>F</td>
<td>p</td>
<td>df†</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
<td>Heat</td>
<td>1,11</td>
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<td>0.2804</td>
<td>1,11</td>
<td>4.91</td>
<td>0.0488</td>
</tr>
<tr>
<td>Precip</td>
<td>1,11</td>
<td>2.50</td>
<td>0.1421</td>
<td>1,11</td>
<td>0.99</td>
<td>0.3407</td>
</tr>
<tr>
<td>Heat x Precip</td>
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<td>2.17</td>
<td>0.1688</td>
<td>1,11</td>
<td>5.56</td>
<td>0.0379</td>
</tr>
<tr>
<td>TFtype‡</td>
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<td>0.1704</td>
<td>1,13</td>
<td>8.69</td>
<td>0.0113</td>
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<tr>
<td>TFtype x Heat</td>
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<td>0.9368</td>
<td>1,13</td>
<td>0.54</td>
<td>0.4757</td>
</tr>
<tr>
<td>TFtype x Precip</td>
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<td>0.11</td>
<td>0.7429</td>
<td>1,13</td>
<td>0.04</td>
<td>0.8481</td>
</tr>
<tr>
<td>TFtype x Heat x Precip</td>
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<td>0.5659</td>
<td>1,13</td>
<td>0.16</td>
<td>0.6920</td>
</tr>
<tr>
<td>Estatus</td>
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</tr>
<tr>
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<td>0.6537</td>
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<td>Estatus x Precip</td>
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<tr>
<td>Estatus x Heat x Precip</td>
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<td>0.4485</td>
</tr>
<tr>
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</tr>
<tr>
<td>TFtype x Estatus x Precip</td>
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<td>0.8980</td>
</tr>
<tr>
<td>TFtype x Estatus x Heat x Precip</td>
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<td>1,15</td>
<td>0.35</td>
<td>0.5619</td>
</tr>
</tbody>
</table>

†Numerator, denominator degrees of freedom
‡Only genotypes CTE45 and NTE19 considered in Analysis 2
§Insufficient degrees freedom and residual error in model to estimate ANOVA results for Soil C and Soil N in PROC GLIMMIX.
Appendix 3. Pearson's correlation coefficients (r) and p-values of Pearson's correlation (p) between fungal and plant parameters for all samples used in this study. Values are not given for correlations where R = 1. Significant p-values (α = 0.05) are denoted with red bold type.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AMF Hyphae</th>
<th>AMF Vesicles</th>
<th>AMF Arbuscules</th>
<th>Total AMF</th>
<th>DSE</th>
<th>Total N</th>
<th>Root C/N</th>
<th>Leaf N</th>
<th>Root P</th>
<th>Shoot C</th>
<th>Shoot N</th>
<th>Shoot P</th>
<th>Shoot N:P</th>
<th>Shoot C:N</th>
<th>Root C</th>
<th>Root N</th>
<th>Root P</th>
<th>Root N:P</th>
<th>Root C:N</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERH</td>
<td>-0.032</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
<td>0.057</td>
<td>-0.048</td>
<td>-0.057</td>
<td>-0.032</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
<td>-0.048</td>
<td>-0.057</td>
<td>-0.032</td>
<td>0.057</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
<td>-0.048</td>
</tr>
<tr>
<td>Total AMF</td>
<td>-0.032</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
<td>0.057</td>
<td>-0.048</td>
<td>-0.057</td>
<td>-0.032</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
<td>-0.048</td>
<td>-0.057</td>
<td>-0.032</td>
<td>0.057</td>
<td>-0.073</td>
<td>-0.246</td>
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<td>-0.048</td>
</tr>
<tr>
<td>DSE</td>
<td>-0.032</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
<td>0.057</td>
<td>-0.048</td>
<td>-0.057</td>
<td>-0.032</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
<td>-0.048</td>
<td>-0.057</td>
<td>-0.032</td>
<td>0.057</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
<td>-0.048</td>
</tr>
<tr>
<td>Root C/N</td>
<td>-0.032</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
<td>0.057</td>
<td>-0.048</td>
<td>-0.057</td>
<td>-0.032</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
<td>-0.048</td>
<td>-0.057</td>
<td>-0.032</td>
<td>0.057</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
<td>-0.048</td>
</tr>
<tr>
<td>Leaf N</td>
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<td>-0.073</td>
<td>-0.246</td>
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<td>0.057</td>
<td>-0.048</td>
<td>-0.057</td>
<td>-0.032</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
<td>-0.048</td>
<td>-0.057</td>
<td>-0.032</td>
<td>0.057</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
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</tr>
<tr>
<td>Root P</td>
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<td>-0.057</td>
<td>-0.032</td>
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<td>-0.048</td>
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<td>-0.032</td>
<td>0.057</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
<td>-0.048</td>
</tr>
<tr>
<td>Root N:P</td>
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<td>-0.246</td>
<td>-0.148</td>
<td>0.057</td>
<td>-0.048</td>
<td>-0.057</td>
<td>-0.032</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
<td>-0.048</td>
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<td>-0.032</td>
<td>0.057</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
<td>-0.048</td>
</tr>
<tr>
<td>Root C:N</td>
<td>-0.032</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
<td>0.057</td>
<td>-0.048</td>
<td>-0.057</td>
<td>-0.032</td>
<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
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<td>-0.057</td>
<td>-0.032</td>
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<td>-0.073</td>
<td>-0.246</td>
<td>-0.148</td>
<td>-0.048</td>
</tr>
</tbody>
</table>

*P-values of correlation (p) and coefficients (r) are shown in bold type, respectively in red or blue. Significant correlations (α = 0.05) are denoted in red.*
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EDUCATION

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PUBLICATIONS


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• Doyle E. Peaslee Award for outstanding graduate student in Plant and Soil Sciences at UK, 2015-2016 academic year winner.

• First place speaker, August UK Integrated Plant and Soil Sciences (IPSS) Graduate Student Research Symposium. Presentation Title: “The effects of multiple aboveground fungal endophyte genotypes on belowground arbuscular mycorrhizal fungal colonization of tall fescue roots, plant nutrients, and soil properties.” 2015

• Second place speaker, Emerging Scientist Competition, American Forage and Grassland Council (AFGC) Annual Meeting in St. Louis, MO. Presentation title: “How does the aboveground fungal endophyte *Epichloë coenophiala* impact root colonization of arbuscular mycorrhizal fungi in tall fescue?” 2015

• Second place speaker, August UK Integrated Plant and Soil Sciences (IPSS) Graduate Student Mini-Symposium. Presentation Title: “How does the aboveground fungal endophyte *Epichloë coenophiala* impact colonization of arbuscular mycorrhizal fungi in tall fescue?” 2014

• First place speaker, Robert F. Barnes C06 Graduate Student Oral Competition, ASA-CSSA-SSSA International Annual Meetings in Long Beach, California. Presentation title: “Grass-Fungal Endophyte Symbiosis Effects on Nitrogen Fixation and Dynamics in a Kentucky Pasture”. 2014