Effects of multiple climate change factors on the tall fescue–fungal endophyte symbiosis: infection frequency and tissue chemistry

Glade B. Brosi1, Rebecca L. McCulley1, Lowell P. Bush1, Jim A. Nelson1, Aimée T. Classen2 and Richard J. Norby3

1Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY 40546-0091, USA; 2Department of Ecology and Evolutionary Biology, University of Tennessee, Knoxville, TN 37996-1610, USA; 3Environmental Science Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6422, USA

Author for correspondence:
Rebecca L. McCulley
Tel: +1 859 2576388
Email: rebecca.mcculley@uky.edu

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Summary

- Climate change (altered CO₂, warming, and precipitation) may affect plant–microbial interactions, such as the Lolium arundinaceum–Neotyphodium coenophialum symbiosis, to alter future ecosystem structure and function.
- To assess this possibility, tall fescue tillers were collected from an existing climate manipulation experiment in a constructed old-field community in Tennessee (USA). Endophyte infection frequency (EIF) was determined, and infected (E+) and uninfected (E−) tillers were analysed for tissue chemistry.
- The EIF of tall fescue was higher under elevated CO₂ (91% infected) than with ambient CO₂ (81%) but was not affected by warming or precipitation treatments. Within E+ tillers, elevated CO₂ decreased alkaloid concentrations of both ergovaline and loline, by c. 30%; whereas warming increased loline concentrations 28% but had no effect on ergovaline. Independent of endophyte infection, elevated CO₂ reduced concentrations of nitrogen, cellulose, hemicellulose, and lignin.
- These results suggest that elevated CO₂, more than changes in temperature or precipitation, may promote this grass–fungal symbiosis, leading to higher EIF in tall fescue in old-field communities. However, as all three climate factors are likely to change in the future, predicting the symbiotic response and resulting ecological consequences may be difficult and dependent on the specific atmospheric and climatic conditions encountered.

Introduction

Atmospheric CO₂ concentrations are increasing and may result in a 1.4–5.8°C increase in global average air temperatures by 2100, with subsequent impacts on regional precipitation patterns (IPCC, 2007). These changes in climate could have significant ramifications for species interactions and the structure and function of ecosystems around the world. One class of species interactions that is likely to respond to climate change and has various ecosystem-level effects are microbial–plant symbioses. For example, recent work has illustrated elevated CO₂ concentrations can increase ectomycorrhizal colonization of some plant species (Garcia et al., 2008) and increase microbial nitrogen fixation in legumes (Rogers et al., 2009) – both of which may result in alterations in nutrient availability, capture and use in the ecosystems where they occur.

An important microbial–plant symbiosis that has received less attention regarding potential climate change response and resulting ecological effects is that of aboveground fungal endophytes in grasses. This type of symbiosis is widespread, occurring in 20–30% of grasses worldwide (Leuchtmann, 1992), and the interactions between grass host and endophyte can range from mutualistic to parasitic (Saikkonen et al., 2006). In species where endophyte symbiosis is thought to be mutualistic, fungal endophytes frequently confer environmental stress tolerance. For example, fungal endophytes can improve plant performance in times of water stress (Kannadan & Rudgers, 2008) and heat stress (Rodriguez et al., 2008), low nutrient availability.
changes in atmospheric \[\text{CO}_2\] interact with endophyte mate change factors. These previous studies evaluated how grass–fungal endophyte symbioses to atmospheric and climate change factors affect the frequency of endophyte infection within tall fescue communities? Will climate change factors interact with the tall fescue–\textit{Neotyphodium} symbiosis to alter tall fescue tissue chemistry? Will climate change factors interact to alter fungal endophyte alkaloid production?

**Materials and Methods**

**Experimental design**

To examine the effects of climate change on the tall fescue–endophyte symbiosis we used an existing multifactor atmospheric and climate change manipulation experiment at Oak Ridge National Laboratory, Tennessee (35°54′12″ N, 84°20′22″ W). The Oldfield Community Climate and Atmospheric Manipulation (OCCAM) experiment ran from 2002 to 2008 and measured the response of a constructed old field plant community to the direct and interactive effects of atmospheric \[\text{CO}_2\] (ambient, ambient +300 ppm), air temperature (ambient, ambient +3°C), and precipitation (dry, wet) (Dermody et al., 2007; Garten et al., 2008). This experiment was a randomized, complete block, split-plot design with three blocks, each containing four plots. Each plot received one of the following treatments: ambient \text{CO}_2–ambient temperature (ACAT); ambient \text{CO}_2–elevated temperature (ACET); elevated \text{CO}_2–ambient temperature (ECAT); elevated \text{CO}_2–ambient temperature (ECAT); and elevated \text{CO}_2–elevated temperature (ECET). Each whole plot was split along the north–south axis into two halves and each half was randomly assigned to one of two precipitation regimes, either ‘dry’ (2 mm H\text{2}O weekly additions) or ‘wet’ (25 mm H\text{2}O weekly additions). Ambient precipitation was excluded with overhead shelters.

Each whole plot was enclosed in a 4 m diameter, 2.2 m high polyvinylchloride open-top chamber. Elevated \text{CO}_2 concentration was maintained by injecting pure \text{CO}_2 into the chamber at ambient +300 ppm. Average \text{CO}_2 concentrations for the experiment were 396 ± 3 ppm in ambient \text{CO}_2 plots and 696 ± 10 ppm in elevated \text{CO}_2 plots.
Temperature treatments were maintained using electric heaters and evaporative coolers, as outlined by Norby et al. (1997). Because chambers can affect temperature within the plots, ambient temperature plots were based on the current temperature data recorded outside the plot and elevated temperature was ambient +3°C (e.g., Norby et al., 1997). Rainfall for the precipitation treatments was collected in the area and applied by hand via metered wands. All treatments started April 2003 and ran continuously until August 2008.

An old-field plant community typical of the southeastern USA (Engel et al., 2009; Kardol et al., 2010a) was chosen for the study. Seven species, consisting of grasses, forbs and legumes were selected: Canada goldenrod (Solidago canadensis L.), red clover (Trifolium pratense L.), sericea lespedeza (Lespedeza cuneata Dum. Cours. G. Don), ribwort plantain (Plantago lanceolata L.), orchardgrass (Dactylis glomerata), broomsedge (Andropogon virginicus), and tall fescue (Lolium arundinaceum L.). Plants were grown from seed (purchased from Ernst Conservation Seeds, Meadville, PA, USA) in a glasshouse in 2002 and then transplanted into the plots in July 2002 in a grid pattern so that individuals were not a neighbor of a conspecific and were c. 18 cm apart. Approximately 170 individuals were planted per 12.6 m² plot.

Sampling and chemical analysis

In July 2008, immediately before the termination of this multiyear project, one tiller was collected from each individual tall fescue plant in every plot (c. 10 tillers per split-plot x 24 split-plots = c. 240 tillers in total). Tillers were kept cold and transported to the laboratory where fungal endophyte (N. coenophialum) infection was determined by an enzyme-linked endophyte-specific immunosorbent assay (Hill et al., 2002). Endophyte infection frequency was calculated as the percentage of infected tillers per plot. Pretreatment (July 2002) endophyte infection data were not available, but there is no reason to think that infection frequencies differed significantly among plots at time zero as plants were all grown from one seed lot. Endophyte infection of pre-2008 harvested tall fescue material could not be determined, as material was not saved in a way to allow for this analysis. July 2008 endophyte-infected and endophyte-free tillers were separated based on assay results into E+ and E− groups for each plot. Tillers were freeze-dried and ground through a 1 mm screen on a Model 4 Wiley Mill (Thomas Scientific Inc., Swedesboro, NJ, USA). Per cent cellulose, hemicellulose, and lignin were determined on 0.5 g subsamples of the E+ and E− tiller groups using the Van Soest (1963) method with an ANKOM fiber analyser (Ankom Technology Inc., Fairport, NY, USA). An additional subsample of the E+ and E− ground material was further homogenized by ball-grinding and subsequently evaluated for carbon (C) and N content using a Flash EA1112 elemental analyser (ThermoFisher Scientific Inc., Waltham, MA, USA).

We determined alkaloid content of E+ tillers only because E− tall fescue does not contain measurable quantities of loline and ergot alkaloids (as verified in our laboratory; see Siegrist et al., 2010). The presence of the Neotyphodium endophyte is required for alkaloid production: tall fescue does not have this physiological capability otherwise (Bush et al., 1997). We measured loline (pyrrolizidine) and ergot (ergopeptide) alkaloids which have been shown to affect insect and mammalian herbivores (Bush et al., 1997). Loline alkaloids – N-formyl loline (NFL), N-acetyl norloline (NANL) and N-acetyl loline (NAL) – were extracted from Wiley-milled plant material with ethanol–methylene chloride (4 : 1, v : v) containing the internal standard quinoline and sodium bicarbonate, and were quantified using a gas chromatograph equipped with a flame ionization detector following the protocol of Blankenship et al. (2001). For ergot alkaloids, a 0.5 g subsample of the Wiley Mill ground tall fescue tiller material was extracted in 10 ml of 80% methanol by mechanical shaking for 2 h. Extract was decanted through a cotton filter in a glass tube and then through a Strata C18–U 55 μm, 70A filter (Phenomenex Inc., CA, USA). The first 2 ml eluant from the Strata filter was discarded and the third milliliter was used for alkaloid determination. A modified high-performance liquid chromatography (HPLC) procedure developed by Yates & Powell (1988), utilizing fluorescence detection following a 5.0 μl injection, was used to quantify ergot alkaloids (ergovaline and ergovalinine). Separation was performed using a Kinetex C18 column (150 × 4.6 mm) (Phenomenex Inc., CA, USA) with 2.6 μm particle size. Solutions used for elution were (A) 0.1 M ammonium acetate–acetonitrile, 97 : 3 v : v and (B) 100% acetonitrile. The elution gradient was: 90 : 10 (A : B) 5.5 min; linear change to 70 : 30 (A : B) during the next 0.5 min; linear change to 56 : 44 (A : B) during the next 15 min; step change to 0 : 100 (A : B) during the next 5 min and step change to 90 : 10 (A : B) and held for 9 min.

Statistical analysis

Data were analysed using a mixed linear model and the method of restricted maximum likelihood (REML, Proc MIXED; SAS Institute, Cary, NC, USA). For parameters measured at the split-plot level (i.e. one data point for each split-plot: endophyte infection frequency and alkaloid concentrations of the E+ tillers), the effects of CO₂, temperature, precipitation, and their interactions were considered fixed effects, and block and the interaction between block, CO₂ and temperature were included as random effects. Most split-plots in the experiment had both E+ and E− tillers, and the litter chemistry analyses were conducted...
separately for these two groups of tillers per split-plot. Thus, infection status was incorporated as a split-split-plot factor in the statistical analysis of the concentrations of C, N, cellulose, hemicellulose, lignin, and C : N ratio. Endophyte status was added as a fixed effect to the split-plot model, and the interaction between block, CO2, temperature and water was added to the split-plot model random effects. Fixed effects were evaluated using the ‘containment’ method in SAS to calculate denominator degrees of freedom. Missing data for split-plots that did not have E− tillers (n = 3) or not enough material to run all analyses created an unbalanced design that reduced degrees of freedom accordingly in these models. Endophyte infection frequencies and concentrations of C, N, hemicellulose, cellulose and lignin were arcsine-square root transformed before analysis so that errors met assumptions of normality and homogeneity of variance.

### Results

#### Endophyte infection frequency and alkaloid concentrations

Elevated CO2 was the predominant climate change factor affecting EIF within the tall fescue community. The EIF was significantly higher under elevated CO2 than with ambient (Tables 1,2), suggesting that elevated CO2 promoted the plant–fungal endophyte mutualism over the 5-yr study. Surprisingly, EIF was not significantly different under altered precipitation, temperature, or any treatment combination (Table 2).

For E+ tall fescue tillers, the effects of CO2, temperature, and altered precipitation on alkaloid concentrations were mixed. Elevated CO2 significantly decreased the total concentrations of both loline and ergot alkaloids (by 26% and 31%, respectively, Tables 1,2), primarily by reducing concentrations of N-formyl loline and ergovaline (data not shown). Conversely, elevated temperature increased loline concentrations by 29–60% but had no effect on ergot alkaloids (Tables 1,2). The wet (vs dry) precipitation treatment led to a reduction in the loline alkaloids, NANL and NAL (c. 29% for both, Table 1), but had no significant (P > 0.05) effect on NFL or total loline alkaloid concentrations (Table 2). For the ergot alkaloids, ergovaline concentration was somewhat reduced in the dry treatment (0.17 mg kg−1 dry vs 0.22 mg kg−1 wet, P = 0.0692, data not shown); however, precipitation treatment had no effect on ergovaline or the combined total ergot alkaloid concentrations (Tables 1,2).

#### Tall fescue tissue chemistry

Elevated CO2 decreased the N concentration of above-ground tall fescue plant material (Table 1) but had no effect on % C (Table 3). Consequently, C : N ratios increased under elevated CO2 conditions (Table 1). Cellulose and hemicellulose, as well as lignin, decreased under elevated CO2 concentrations (Tables 1,3). The wet treatment increased concentrations of cellulose and hemicellulose compared with the dry treatment (Table 1), but the percentage of C decreased by 0.5% (Table 3; data not shown).

The presence of Neotyphodium had no significant impact on the percentage of N, or C : N, or the percentage of cellulose and hemicellulose, or of lignin (Table 3). However, endophyte infection reduced the percentage of C in tall fescue tillers (42.60% E− vs 41.80% E+), but only under elevated CO2 (P = 0.0077 for CO2 × endophyte; Table 3).

### Table 1 Response of endophyte-related parameters and tall fescue tissue chemistry to changes in [CO2], air temperature and water availability

<table>
<thead>
<tr>
<th>Endophyte-related parameters</th>
<th>Tall fescue tissue chemistry</th>
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<tbody>
<tr>
<td>Endophyte infection frequency (%)</td>
<td>NFL (mg kg−1)</td>
</tr>
<tr>
<td>CO2</td>
<td></td>
</tr>
<tr>
<td>Ambient 81 ± 3</td>
<td>784 ± 32</td>
</tr>
<tr>
<td>Elevated 81 ± 2</td>
<td>556 ± 39</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
</tr>
<tr>
<td>Ambient 86 ± 3</td>
<td>612 ± 46</td>
</tr>
<tr>
<td>Elevated 86 ± 3</td>
<td>727 ± 46</td>
</tr>
<tr>
<td>Water availability</td>
<td></td>
</tr>
<tr>
<td>Dry 86 ± 3</td>
<td>644 ± 53</td>
</tr>
<tr>
<td>Wet 86 ± 3</td>
<td>695 ± 45</td>
</tr>
</tbody>
</table>

Endophyte infection frequency, endophyte-associated alkaloids (N-formyl loline, NFL; N-acetyl norloline, NALN; N-acetyl loline, NAL; Total lollines, and Total ergots; from endophyte-infected tillers only as endophyte-free tillers do not have measurable quantities of these compounds) and tall fescue tissue chemistry parameters (%N, C : N ratio, and % cellulose and hemicellulose, lignin). Values are mean ± 1 SE. Bold values indicate significant treatment effects (P < 0.05).
Discussion

Because of the well-documented benefits of *Neotyphodium* infection for tall fescue under abiotic stress (Arechavaleta *et al.*, 1989; Malinowski, 1999; Rahman & Saiga, 2007), we predicted that after 5 yr of climate treatments the endophyte infection frequency (EIF) of tall fescue would be higher under elevated temperature and under 'dry' precipitation treatments. However, we found no support for this hypothesis. We observed no significant differences in the EIF of tall fescue between wet vs dry treatments or under elevated vs ambient heat (or in the interaction of these two treatments). Elevated CO₂ was the only treatment that produced a significant change in EIF of the tall fescue.
community, and it also did not interact with the warming or precipitation treatments. This elevated CO₂ response is interesting given that tall fescue biomass did not respond to increased atmospheric [CO₂] over the course of this experiment. Rather, tall fescue and lespedeza, the dominant species by the end of the experiment, responded primarily to the watering treatment, both increasing under wet conditions (Kardol et al., 2010a,b). The plant community, as a whole, did not differ between ambient and elevated [CO₂] (Engel et al., 2009); therefore, contrary to previous work showing that high EIF enhances the competitive ability and dominance of tall fescue in plant communities (Clay & Holah, 1999), the fungal–grass symbiosis appears to have had little direct effect on the plant community dynamics of this experiment. It is possible that only a 10% increase in endophyte-infection rates between the CO₂ treatments, while significant, was not enough to affect the surrounding plant community, especially as tall fescue in both ambient and elevated CO₂ plots was > 80% infected with Neotyphodium.

Endophyte infection frequency within a tall fescue community might change over time because of differential survival or recruitment of E+ or E– individual tillers or to alterations in the efficacy of vertical transmission. Increased EIF under elevated CO₂ may have arisen from increased atmospheric [CO₂] directly altering the plant and fungal endophyte physiology and transmission rates or indirectly altering the surrounding environment in ways that promoted the symbiosis (e.g. competition for nutrients, increasing herbivory levels, etc.). Previous studies have demonstrated that the physiology of grass–Neotyphodium symbioses can be sensitive to elevated CO₂ (Newman et al., 2003; Hunt et al., 2005), and similar to these studies, we observed significant alterations in tiller percentage of N, C : N ratio and percentage of cellulose, hemicellulose and lignin in response to elevated CO₂ concentrations. We did not find significant endophyte effects or interactions with CO₂ for these tissue chemistry parameters, as has been previously reported; however, the observed reduction in concentrations of both total loline and ergot alkaloids in E+ tall fescue tillers under elevated vs ambient CO₂ supports the idea that plant–fungal physiology was affected by this atmospheric manipulation. It is not known whether or how elevated CO₂ might directly affect fungal physiology or endophyte vertical transmission within the plant.

Previous work has shown that N availability can also interact with [CO₂] and endophyte infection to alter tall fescue growth and tissue chemistry (Newman et al., 2003; Chen et al., 2007). Two of the seven species included in this experiment were N-fixers, and symbiotic N-fixation rates were relatively high (4.6–12 g N m⁻² yr⁻¹; contributing c. 50% of the N contained in aboveground biomass in a plot in any given year) and largely unresponsive to the climate treatments (Garten et al., 2008). Thus, N availability may have been similar across the climate treatments. The significant reduction in tiller foliar percentage N under elevated CO₂ suggests that some N limitation did occur in this treatment and, interestingly, this is the only treatment where a significant endophyte effect and significant endophyte by treatment interaction was observed on a tissue chemistry parameter (% C), perhaps suggesting, as others have found, that N availability will play a role in determining endophyte effects on tall fescue tissue chemistry under future environmental conditions. Most previous work has shown that endophyte effects on tall fescue tissue chemistry are more pronounced under high N availability (Belesky et al., 1984; Lyons et al., 1990; Newman et al., 2003), and while symbiotic N-fixation rates measured during this project were relatively high, they were much lower than typical fertilizer N additions, such as those employed in the previous work on the topic. Nitrogen availability is likely to be an additional factor that will affect the nature and degree of the tall fescue–Neotyphodium symbiotic response to future climate change.

Previous and concurrent studies at the site have reported that elevated CO₂ effects on herbivorous insect and nematode abundances were relatively minor compared with those observed in the warming and wet treatments (Villalpando et al., 2009; Kardol et al., 2010b). If high herbivory levels confer a competitive advantage to E+ tall fescue – enough to promote the survival or recruitment of E+ over E– tillers during the 5-yr experiment – increased EIF should have been observed in the heated and wet treatments (where abundances of herbivorous insects and nematodes were greatest, respectively), but this is not what we observed. It is possible that the open-top chambers used in this experiment may have reduced insect and rodent herbivory (overall or in species-specific ways) such that this well-known mechanism through which EIF within tall fescue can alter the surrounding plant community composition (Clay et al., 2005) was unable to exert as much influence as is typically encountered in old field environments.

The production of toxic alkaloids by Neotyphodium was clearly affected by changes in [CO₂], as also reported by Hunt et al. (2005), and climate, and as these alkaloids are known to have negative effects on the herbivores that consume them (Kindler et al., 1991; Thompson et al., 2001), such changes might lead to significant ecological consequences. Elevated CO₂ decreased both total ergot and loline alkaloid concentrations by c. 30%; thus, while more individual tall fescue tillers were endophyte-infected under elevated CO₂, the plant material produced by these individuals had lower concentrations of these toxic compounds than E+ tillers from other treatments. Given that alkaloids are often invoked as the mechanism by which fungal endophytes alter ecological processes, it would be interesting to know whether these trends resulted in a net increase, decrease or no net change in the quantity of alkaloids in the plots.
Unfortunately, these calculations require data that were not collected at the time of this study.

Although elevated temperature did not affect EIF, total loline concentrations increased 28.5% in the E+ material in this treatment. Similarly, concentrations of two types of loline alkaloids, NAL and NAL, were significantly higher in the dry vs wet treatment. The increased concentrations of loline alkaloids in tall fescue material grown under elevated temperature may be related to increased herbivory from the greater insect abundance associated with this treatment (Villalpando et al., 2009). However, greater alkaloid concentrations in the elevated temperature and the dry precipitation treatment may also be linked to the ability of E+ tall fescue to withstand drought stress and is consistent with previous findings on this topic (Arechavaleta et al., 1992; Schardl et al., 2007).

Drought stress (low water and/or high heat) may interact with the plant–fungal endophyte symbiosis to increase alkaloid production in tall fescue (Schardl et al., 2004). As alkaloids are thought to be the primary mechanism by which endophyte infection alters herbivory, plant competition and ecosystem processes such as litter decomposition (Rudgers et al., 2004) in tall fescue dominated systems, we might predict that under drier and hotter conditions these alkaloid-related effects on ecological processes are likely to be larger, not smaller, but elevated CO$_2$ might mitigate or negate these potential effects to some degree. We cannot explain why, despite having higher concentrations of alkaloids in E+ material and the often-cited advantages of fungal endophyte infection and alkaloid production to tall fescue, we observed no differences in EIF in the tall fescue communities in the heated and dry treatments but instead observed increased EIF under elevated CO$_2$ (but with lower alkaloid concentrations). It is also possible that the implementation of the warming and dry treatments was not conducive to promoting the drought advantages thought to be conferred by the endophyte to tall fescue. However, these treatments were significant enough to produce distinct soil temperature and moisture regimes (Dermody et al., 2007) to which the plant community composition responded (e.g. tall fescue biomass was reduced by 30% in the dry treatment compared with the wet; Kardol et al., 2010a), and the significant effects on loline alkaloids also suggest the warming and dry treatments were inducing physiological responses in the plant–fungal symbiosis. More work exploring potential mechanisms underlying these results seems warranted.

Independent of infection with Neotyphodium, elevated CO$_2$ concentrations altered tall fescue tissue chemistry in some expected ways. For example, elevated CO$_2$ concentration increased the C : N ratio and decreased %N of tall fescue, which has been demonstrated widely in many other plant species and CO$_2$ enrichment studies (Leakey et al., 2009). We also found a significant decrease in per cent cellulose and hemicellulose as well as lignin when tall fescue was grown in elevated CO$_2$ concentrations. Previous work on tall fescue has shown no significant main effect of elevated CO$_2$ on either hemicellulose and cellulose or lignin, but we did find that lignin was decreased 14% under elevated CO$_2$ with the addition of nitrogen (Newman et al., 2003). The N availability at our site was probably much lower than that of the N addition employed by Newman et al. (2003), so it is interesting that we observed a greater response (43% reduction in lignin) to elevated CO$_2$. From an herbivore perspective, decreased concentrations of cellulose, hemicellulose and lignin would increase palatability. However, the subsequent decrease in the percentage of N and the increase in C : N ratio under elevated CO$_2$ could offset the impact of CO$_2$ on tall fescue tissue chemistry, resulting in no change to palatability. Fewer, but significant effects of the precipitation treatment, endophyte infection and interactions between CO$_2$ and endophyte infection on % C suggest that tall fescue tissue chemistry will be sensitive to future changes in atmospheric and climatic conditions and somewhat dependent on endophyte status.

The various effects of the climate treatments on tissue chemistry (both alkaloids and %N, cellulose, etc.), coupled with the unexpected result that EIF was not altered in the elevated temperature and/or dry treatment but did increase under elevated CO$_2$, clearly indicate that the tall fescue–fungal endophyte symbiosis and the resulting ecological effects of this relationship will be affected by future atmospheric and climatic conditions. To our knowledge this is the first field experiment to quantify alterations in fungal endophyte–grass symbiosis resulting from multiple atmospheric and climate changes. While the results suggest that the tall fescue–fungal endophyte symbiosis is particularly sensitive to atmospheric [CO$_2$], we know that all three factors ([CO$_2$], temperature, and water availability) are likely to be altered in coming years. Therefore, the response of this symbiosis in planta and within the ecological community will depend largely on the specific environmental conditions encountered. Given the extensive acreage of tall fescue worldwide and the fact that the ecological effects of this grass–fungal endophyte symbiosis have been observed at population, community, and ecosystem-scales (Omacini et al., 2005), understanding the response of tall fescue and its endophytic fungi Neotyphodium to climate change may be important in predicting not only the responses of grazing livestock and other herbivores but also that of ecological processes such as litter decomposition and nutrient cycling.

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