The advantages of endophyte-infected over uninfected tall fescue in the growth and pathogen resistance are counteracted by elevated CO₂

Wei Chen1, Hui Liu1, Wurihan1, Yubao Gao1, Stuart D. Card2 & Anzhi Ren1

Atmospheric CO₂ concentrations are predicted to double within the next century. Despite this trend, the extent and mechanisms through which elevated CO₂ affects grass-endophyte symbionts remain uncertain. In the present study, the growth, chemical composition and pathogen resistance of endophyte-infected (E⁺) and uninfected (E⁻) tall fescue were compared under elevated CO₂ conditions. The results showed that the effect of endophyte infection on the growth of tall fescue was significantly affected by elevated CO₂. Significant advantage of E⁺ over E⁻ tall fescue in tiller number, maximum net photosynthetic rate and shoot biomass occurred only under ambient CO₂. With CO₂ concentration elevated, the beneficial effect of endophyte infection on the growth disappeared. Similarly, endophyte infection reduced lesion number and spore concentration of Curvularia lunata only under ambient CO₂. These results suggest that the beneficial effect of endophyte infection on the growth and pathogen resistance of tall fescue could be counteracted by elevated CO₂. An explanation for the counteraction may be found in a change in photosynthesis and nutritive quality of leaf tissue.

Atmospheric carbon dioxide (CO₂) is expected to rise from a current ambient concentration of 390 ppm to between 550 and 1000 ppm by the year 2100. CO₂ enrichment in the atmosphere in general stimulates photosynthetic activity and growth of C₃ plants. CO₂ enrichment has also been shown to change plant resource allocation, especially plant C:N ratio. The carbon-nutrient balance (CNB) hypothesis predicts that carbon products in excess of those needed for primary metabolic functions will result in increased carbon-based secondary metabolites and subsequently decreased N-based secondary metabolites. Such alterations in plant primary and secondary metabolism are expected to alter the availability of photosynthates and defensive compounds for plant-associated microbes, modifying plant-microbial interactions such as plant-rhizobial symbiosis, mycorrhizae, and plant-endophyte complexes.

Endophytic fungi within asymptomatic aerial tissues of plants represent a ubiquitous component of terrestrial plant communities. Among them, the symbiosis between cool season grasses and Epichloë endophytes is most common and considered to have important ecological implications. In the symbiosis, the host grasses provide photosynthates and nutrients to the endophytes, and in turn, the endophytes can benefit grasses through increased growth and by providing tolerance to abiotic and biotic stresses. Increasing evidence shows that the endophyte-host interaction depends in many cases, on resource availability. In fact, many of the studies that have found improved growth and resistance in endophyte-infected grasses were done under benign conditions of moderate to high soil nutrient availability.

Many studies have shown that elevated CO₂ increases mycorrhizal colonization of roots and alters plant – mycorrhizal interactions. Similar to mycorrhiza, fungal endophytes depend on carbon and energy provided by their host plants. However, up to now, studies examining responses of grass-endophyte associations to elevated CO₂ are limited. In the pioneering study, Marks and Clay found in perennial ryegrass (Lolium perenne, a...
C₃ plant) and purpletop grass (Tridens flavus, a C₄ plant), the growth of endophyte-infected (E⁺) and uninfected (E⁻) plants responded similarly to CO₂ enrichment. Also in perennial ryegrass, Hunt, et al. reported that E⁺ biomass tended to be greater than E⁻ plants only at elevated CO₂, and they further found that E⁻ plants had 40% lower concentrations of soluble protein under elevated CO₂ than under ambient CO₂, but this CO₂ effect on soluble protein was absent in E⁺ plants. In tall fescue (Lolium arundinaceum), Newman, et al. did not find interaction between CO₂ concentration and endophyte infection in the growth, but they found soluble crude protein concentration increased under elevated CO₂ for E⁻ plants but not for E⁺ plants. Ryan, et al. reported that endophyte-derived alkaloids decreased in response to elevated CO₂. Taken together, the effects of endophyte infection on herbage quality as well as defensive chemistry can be affected by elevated CO₂. Therefore, the endophyte-induced herbivore and pathogen resistance of the host are likely to be impacted by elevated CO₂ in the atmosphere.

Recently, the effect of Epichloë endophyte infection on pathogen resistance has been extensively investigated. The pioneering research by Shimanuki and Sato demonstrated that timothy plants (Phleum pratense) infected by Epichloë typhina were resistant to the fungus Cladosporium phlei. In in vitro investigations, White and Cole, Siegel and Latch, and Christensen found that Epichloë isolates inhibited the growth of pathogenic fungi, only the antifungal activity of endophytes differed between the isolates. In in planta investigations, the positive effect of endophyte infection on pathogen resistance of the host grass has been observed in tall fescue, ryegrass, and other native grasses. Certainly, endophytes do not always improve disease resistance of the host. Negative and neutral effects have also been reported. In our previous study, we found that endophyte could enhance pathogen resistance of Leymus chinensis, and this endophytic benefit was strengthened by drought treatment. These different reports suggest that the interactions between endophytes and pathogens are complex, and may be affected by species difference as well as environmental factors such as elevated CO₂ concentration in the atmosphere.

In the present study, E⁺ and E⁻ tall fescue were planted under contrasting CO₂ availability regimes to test the effect of the endophyte infection and CO₂ concentration on the performance in terms of growth, chemical composition and pathogen resistance of tall fescue. Specifically, we addressed the following questions: (1) does endophyte infection improve growth and pathogen resistance of the grass host? (2) does elevated CO₂ affect growth and pathogen resistance pattern of tall fescue – endophyte associate? If this is the case, (3) what is the mechanism involved might be?

## Results

### Plant height, leaf number and tiller number.
Plant height was only significantly affected by N availability (Table 1), and N supply increased plant height (HN = 54.19 ± 4.80; LN = 31.61 ± 2.15; cm). Leaf number was significantly increased by elevated CO₂ concentration, N supply as well as endophyte infection (EC = 72 ± 28.6, AC = 63 ± 22.4; HN = 90 ± 15.3; LN = 45 ± 8.3; E⁺ = 75 ± 25.1, E⁻ = 60 ± 24.7). Tiller number was significantly affected by interaction between CO₂ concentration and endophyte infection (Table 1, Fig. 1). Under ambient CO₂ condition, tiller number of E⁺ was significantly more than that of E⁻, but under elevated CO₂ condition, no significant difference occurred.

### Maximum net photosynthetic rate and biomass.
Maximum net photosynthetic rate and shoot biomass were significantly affected by three-way interaction among CO₂ concentration, N supply and endophyte infection (Table 1). Only under ambient CO₂ and high N condition, both maximum net photosynthetic rate and shoot biomass were greater in E⁺ than in E⁻ plants (Fig. 2).

### Leaf carbon, nitrogen and C:N ratio.
Leaf C concentration was significantly affected by N supply and endophyte infection (Table 1). Leaf N concentration was significantly affected by three-way interaction among CO₂ concentration, N supply and endophyte infection. Leaf N concentration of E⁺ plants was lower than that of E⁻ plants only under ambient CO₂ and high N condition (Fig. 3a). Both elevated CO₂ concentration and endophyte infection significantly improved leaf C:N ratio (Fig. 3b,c).

---

### Table 1. Three-way ANOVA for growth characters of endophyte-infected (E⁺) or uninfected (E⁻) Festuca arundinacea under various CO₂ and nitrogen conditions. Significant P-values are in bold.

|                           | F  | P  | F  | P  | F  | P  | F  | P  | F  | P  | F  | P  | F  | P  | F  | P  |
|---------------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Plant height              |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| E⁺                        | 0.10 | 0.76 | 39.94 | 0.00 | 33.76 | 0.00 | 3.25 | 0.08 | 24.27 | 0.00 | 2.59 | 0.12 | 0.16 | 0.69 | 4.22 | 0.05 |
| E⁻                        | 0.96 | 0.34 | 15.96 | 0.00 | 11.19 | 0.00 | 347.42 | 0.00 | 433.83 | 0.00 | 11.03 | 0.00 | 2.03 | 0.16 | 0.13 | 0.72 |
| N × C                     | 0.50 | 0.48 | 5.75 | 0.00 | 1.52 | 0.23 | 6.65 | 0.02 | 13.55 | 0.00 | 0.16 | 0.69 | 0.21 | 0.65 | 0.14 | 0.71 |
| E × N                     | 0.40 | 0.85 | 0.05 | 0.83 | 0.28 | 0.60 | 13.16 | 0.00 | 8.72 | 0.01 | 2.38 | 0.13 | 0.90 | 0.35 | 1.71 | 0.20 |
| C × N                     | 0.31 | 0.58 | 14.82 | 0.00 | 9.43 | 0.00 | 0.03 | 0.87 | 269.27 | 0.00 | 8.33 | 0.01 | 0.03 | 0.86 | 0.51 | 0.48 |
| E × C × N                 | 0.11 | 0.75 | 1.19 | 0.28 | 0.52 | 0.47 | 4.12 | 0.05 | 7.65 | 0.01 | 0.84 | 0.37 | 1.58 | 0.22 | 0.94 | 0.34 |

SCIENTIFIC REPORTS | 7:6952 | DOI:10.1038/s41598-017-07183-y
Lesion number and spore concentration of the pathogen. Both lesion number and pathogen spore concentration were significantly affected by the interaction between CO$_2$ concentration and endophyte infection (Table 2). Under ambient CO$_2$ concentration, endophyte infection reduced lesion number and pathogen spore concentration of the host leaves when exposed to *Curvularia lunata*. Elevated CO$_2$ significantly improved pathogen resistance of both E$^+$ and E$^-$ plants. However, no difference occurred in either lesion number or pathogen spore concentration between E$^+$ and E$^-$ plants under elevated CO$_2$ (Fig. 4). That is to say, the advantage in pathogen resistance of E$^+$ over E$^-$ plants was alleviated by elevated CO$_2$.

Soluble sugar and amino acids. Soluble sugar concentration was significantly affected by CO$_2$ concentration and endophyte infection (Table 2). Elevated CO$_2$ significantly increased soluble sugar concentration while endophyte infection significantly decreased soluble sugar concentration of tall fescue (Fig. 5).

Because the responses of the 17 amino acids that were measured were not independent, after measurement, we used a PCA to reduce the number of amino acid response variables to a new set of composite variables. To facilitate interpretation of the principal components, we subjected the first four principal components to factor...
rotation and retained four rotated factors (RF1, RF2, RF3, and RF4, which accounted for 82.61% of the total variance) (Fig. 6). As the values of the rotated factor increased, the variables that load heavily and positively (loading $\geq +0.5$) also increased, while the variables that load heavily but negatively (loading $\leq -0.5$) decreased. The standardized univariate responses of these variables are shown in Fig. 7 to facilitate the interpretation of the multivariate responses and to allow a closer inspection of the variables loading heavily onto RF1, RF2, RF3, and RF4.

Six amino acids, Asp, Glu, Ser, Thr, Ala and Gly loaded heavily and positively onto RF1; five amino acids, Pro, Arg, Ala, Val and Gly loaded positively onto RF2; four amino acids, Lys, Leu, Ile and Val loaded positively onto RF3 (Fig. 6). The interaction between elevated CO$_2$ and pathogen inoculation significantly affected RF1, RF2 and RF3 (Table 2). In P- group, elevated CO$_2$ tended to enhance RF1, RF2 and RF3, but significant effect only occurred on RF1. In P+ group, elevated CO$_2$ increased RF2, decreased RF3 but had no effect on RF1 (Fig. 7). Cys loaded heavily and positively onto RF4, and Phe loaded heavily but negatively onto RF4 (Fig. 6). Endophyte infection significantly decreased RF4 (Table 2, Fig. 7).

Lignin accumulation. Lignin concentration was significantly affected by interaction between CO$_2$ concentration and pathogen inoculation (Table 2). Pathogen inoculation resulted in lignin accumulation in the leaf of tall fescue under ambient CO$_2$ concentration, and this trend was further strengthened by elevated CO$_2$ concentration (Fig. 8). Lignin concentration was significantly affected by interactions among CO$_2$ concentration, endophyte infection and pathogen inoculation. Only under ambient CO$_2$ and pathogen inoculation condition, lignin concentration of the leaf was greater in E+ than in E- plants (Fig. 8).

Discussion

Plant growth response. The effects of elevated CO$_2$ on growth of plants, especially C$_3$ plants, have been widely studied, but most published papers on plant response to elevated CO$_2$ fail to even state the endophyte status of their plant material. In the pioneering study, Marks and Clay$^{29}$ found no significant interactions between CO$_2$
enrichment and endophyte infection on the growth of perennial ryegrass. Similar results have been reported by Newman, et al.9 in tall fescue. In contrast, in the present study, we found a significant endophyte infection × CO2 interaction for tiller number, maximum net photosynthetic rate and shoot biomass. We found that growth advantage of E+ over E− plants occurred only under high N and ambient CO2 conditions. Under high N conditions, elevated CO2 improved shoot growth of both E+ and E− plants, but the growth advantage of E+ disappeared under elevated CO2. That is to say, elevated CO2 counteracted the beneficial effect of endophyte infection on the growth of the host. Although significant endophyte infection × N supply × CO2 interaction for growth response has not been reported, this result is consistent with most published reports in that growth advantage of E+ plants occurred under high N conditions 9, 23, 30, 49, and consist with the results in tall fescue that no significant difference appeared in growth between E+ and E− plants under elevated CO2 9, 50.

In tall fescue, Brosi, et al.51 found that endophyte infection frequency was significantly higher under elevated CO2 compared to ambient; and Ryan, et al.30 found that endophyte concentration increased under elevated CO2. If fungal concentration was correlated with vegetative vigor of the host plant directly28, elevated CO2 may promote the plant-fungal endophyte mutualism. In the present study, we did find elevated CO2 improve the growth of E+ plants, but elevated CO2 improve the growth of E− plants in a higher degree, and thus a significant growth difference between E+ and E− plants did not exist anymore under elevated CO2. This phenomenon might be related to photosynthetic ability of tall fescue. Tall fescue belongs to a C3 grass. Because of the lack of CO2 concentrating ability, at ambient CO2, its carboxylation function of Rubisco is thought to be limited by CO2. With CO2 concentration in the air increasing, its photosynthetic rate will increase3, 52, 53. Under ambient CO2, photosynthetic ability of grasses can be improved by endophyte infection9, 54–56. Under elevated CO2, the carboxylation function

| Lesion number | Spore concentration | Soluble sugar concentration | Lignin concentration | RF1 | RF2 | RF3 | RF4 |
|---------------|---------------------|-----------------------------|----------------------|-----|-----|-----|-----|
| F  | P  | F  | P  | F  | P  | F  | P  | F  | P  |
| Endophyte (E) | 10.52 | 0.01 | 137.45 | 0.00 | 13.60 | 0.00 | 1.47 | 0.23 | 0.00 | 0.99 | 1.23 | 0.28 | 0.81 | 0.38 | 4.19 | 0.05 |
| CO2 (C)      | 176.09 | 0.00 | 1662.69 | 0.00 | 16.44 | 0.00 | 14.40 | 0.00 | 12.07 | 0.00 | 28.94 | 0.00 | 0.93 | 0.34 | 0.00 | 0.96 |
| Pathogen (P) | — | — | — | — | 1.03 | 0.32 | 282.45 | 0.00 | 0.17 | 0.68 | 0.61 | 0.44 | 2.08 | 0.16 | 1.03 | 0.32 |
| E × C             | 19.57 | 0.00 | 157.03 | 0.00 | 0.09 | 0.76 | 1.79 | 0.19 | 0.11 | 0.75 | 0.11 | 0.74 | 0.23 | 0.64 | 3.57 | 0.07 |
| E × P             | — | — | — | — | 0.01 | 0.93 | 0.88 | 0.56 | 0.03 | 0.88 | 0.09 | 0.77 | 0.26 | 0.61 | 0.39 | 0.54 |
| C × P             | — | — | — | — | 0.00 | 0.98 | 14.30 | 0.00 | 0.04 | 0.02 | 12.10 | 0.00 | 8.35 | 0.01 | 0.00 | 0.98 |
| E × C × P           | — | — | — | — | 0.45 | 0.51 | 3.91 | 0.05 | 0.02 | 0.89 | 0.05 | 0.83 | 0.69 | 0.41 | 0.56 | 0.46 |

Table 2. Three-way ANOVA for pathogen and physiological indices of endophyte-infected (E+) or uninfected (E−) Festuca arundinacea under various CO2 and pathogen conditions. Significant P-values are in bold.

Figure 4. Lesion number (a) and pathogen spore concentration (b) of endophyte-infected (E+) or uninfected (E−) Festuca arundinacea under elevated CO2 (EC) and ambient CO2 (AC) treatments. * Meant significant difference at 0.05 level.
of Rubisco in tall fescue might be near saturation, and the effect of endophyte infection on photosynthesis might be negligible. That is to say, elevated CO2 might counteract the beneficial effect of endophyte infection in photosynthesis and thus biomass to the host plants.

Independent of endophyte infection, elevated CO2 altered tall fescue tissue chemistry in some expected ways3, 57–59, such as increasing carbohydrates (here soluble sugar concentration), decreasing N concentration and thus increasing C:N ratio. As for amino acid concentrations, studies have reported both positive60, 61 and negative30, 62 effects of elevated CO2 on amino acids. In the present study, elevated CO2 tended to enhance the concentration of 12 out of 17 amino acids tested. From an herbivore perspective, increased concentrations of soluble sugar and amino acids would increase palatability63, 64. However, the subsequent decrease in the percentage of N and the increase in C:N ratio under elevated CO2 could offset this impact62, 65.

Endophyte infection has been described to result in a reduction of nitrogenous compounds in tall fescue66, 67 and ryegrass8. In the present study, we found that endophyte infection significantly decreased the soluble sugar concentration, leaf N and increased C:N ratio of the host grass. Endophyte infection had no effect on most amino acids tested, except decreased RF4. Although we found no interaction between CO2 and endophyte on soluble sugar, amino acids concentration and C:N ratio, similar to the results from Ryan, et al.30, we did find significant interaction between CO2 and endophyte infection on leaf N concentration. Under ambient CO2 and high N conditions, E+ plants had smaller leaf N concentration than E− plants. With CO2 elevated, however, no difference between E+ and E− plants occurred. Here, both elevated CO2 and endophyte infection can decrease leaf N concentration, but the decreasing degree resulted from CO2 was even larger. Under high N conditions, elevated CO2 resulted in 54.5% less while endophyte infection resulted in 20.8% less in leaf N concentration. Alkaloids are considered to contribute to defense. Although we did not measure alkaloids in the present study, both Ryan, et al.30 and Brosi, et al.51 in tall fescue found that alkaloid production decreased with CO2 concentration elevated. Ryan, et al.30 further suggested that plants where the C:N ratio was highest would have the lowest alkaloid per unit endophyte concentrations. All these results suggest that CO2 enrichment might buffer the effect of endophyte infection on the N-metabolism of host plants.

**C and N metabolism.** Independent of endophyte infection, elevated CO2 altered tall fescue tissue chemistry in some expected ways3, 57–59, such as increasing carbohydrates (here soluble sugar concentration), decreasing N concentration and thus increasing C:N ratio. As for amino acid concentrations, studies have reported both positive60, 61 and negative30, 62 effects of elevated CO2 on amino acids. In the present study, elevated CO2 tended to enhance the concentration of 12 out of 17 amino acids tested. From an herbivore perspective, increased concentrations of soluble sugar and amino acids would increase palatability63, 64. However, the subsequent decrease in the percentage of N and the increase in C:N ratio under elevated CO2 could offset this impact62, 65.

Endophyte infection has been described to result in a reduction of nitrogenous compounds in tall fescue66, 67 and ryegrass8. In the present study, we found that endophyte infection significantly decreased the soluble sugar concentration, leaf N and increased C:N ratio of the host grass. Endophyte infection had no effect on most amino acids tested, except decreased RF4. Although we found no interaction between CO2 and endophyte on soluble sugar, amino acids concentration and C:N ratio, similar to the results from Ryan, et al.30, we did find significant interaction between CO2 and endophyte infection on leaf N concentration. Under ambient CO2 and high N conditions, E+ plants had smaller leaf N concentration than E− plants. With CO2 elevated, however, no difference between E+ and E− plants occurred. Here, both elevated CO2 and endophyte infection can decrease leaf N concentration, but the decreasing degree resulted from CO2 was even larger. Under high N conditions, elevated CO2 resulted in 54.5% less while endophyte infection resulted in 20.8% less in leaf N concentration. Alkaloids are considered to contribute to defense. Although we did not measure alkaloids in the present study, both Ryan, et al.30 and Brosi, et al.51 in tall fescue found that alkaloid production decreased with CO2 concentration elevated. Ryan, et al.30 further suggested that plants where the C:N ratio was highest would have the lowest alkaloid per unit endophyte concentrations. All these results suggest that CO2 enrichment might buffer the effect of endophyte infection on the N-metabolism of host plants.

**Figure 5.** Soluble sugar concentration of *Festuca arundinacea* under different endophyte (a) and CO2 treatments (b). *Mean significant difference at 0.05 level.
Figure 6. Loadings for each individual amino acid of *Festuca arundinacea* onto the first four rotated factors (RF). The individual amino acids loading heavily either positively (loading ≥ +0.5) or negatively (loading ≤ −0.5) are highlighted in black.

Pathogen resistance. In terms of the disease resistance of endophyte infection on the host, the beneficial effects of endophyte infection have been reported in perennial ryegrass, tall fescue and native grasses. Pańka, et al. observed stronger susceptibility of E− tall fescue to *Rhizoctonia zeae* than E+ counterparts. A significant increase in resistance to dollar spot disease, caused by *Sclerotinia homoeocarpa*, has also been observed in *Festuca rubra*. In the present study, we found that endophyte infection improved pathogen resistance of tall fescue, but the significant effect occurred only under ambient CO2 concentration.

In studies examining plant response to fungal disease under elevated CO2, disease incidence and severity are variable, from decreased, unchanged to increased. When endophyte infection was considered, up to now, we found no report on its contribution to the host grasses under elevated CO2. In the present study, we found that disease severity of both E+ and E− plants decreased under elevated CO2.

The interesting result in the present study is that the advantage of E+ over E− plants in pathogen resistance under ambient CO2 disappeared with CO2 elevated. One possible explanation might be that the nutritive quality of leaves is responsible for pathogen development. Thompson and Drake found positive correlations existed between plant N concentration and disease severity. And this correlation has been proved by McElrone, et al. and Plessl, et al. In the present study, the main effects of elevated CO2 and endophyte infection were similar on reducing leaf N concentration and decreasing pathogen severity, but the contribution of elevated CO2 was even bigger. So it might be larger degree reduction of N concentration resulted from elevated CO2 that cover up the role of endophyte infection on N concentration and thus pathogen resistance. In the present study, we further found that both elevated CO2 and endophyte infection resulted in lignin accumulation in tall fescue after pathogen inoculation. Lignin is one of important phenolic compounds, whose deposition is believed to play a crucial role in barricading the pathogen from invading the plant through physical exclusion. In the present study, lignin accumulation went along with a decrease in susceptibility and might be a factor contributing to pathogen resistance.

Our results shed some light of the effects of elevated CO2 on the mutualistic relationship between a grass and a fungus. Besides CO2 concentrations, other factors such as temperature and water availability are likely to be altered in coming years. Therefore, the response of grass-endophyte symbiosis to pathogens will be more complex and 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, understanding the response of tall fescue and its endophytic fungi to climate change may be important in predicting not only the responses of pathogens, but also grazing herbivores and ecological processes such as litter decomposition and nutrient cycling.
Conclusions
Our experiments provided evidence that endophyte infection improved the growth of tall fescue, but this benefit was affected by elevated CO₂ and N supply. Only under ambient CO₂ and high N conditions, both maximum net photosynthetic rate and shoot biomass were greater in E⁺ than in E⁻ plants. With CO₂ concentration elevated, the beneficial effect of endophyte infection on the growth disappeared. Similarly, endophyte infection can enhance resistance of tall fescue towards *Curvularia lunata* only under ambient CO₂. Elevated CO₂ counteracted the beneficial effect of endophyte infection on the growth and pathogen resistance of the host grass.

Materials and Methods
Plant material. Endophyte-infected (E⁺) seeds of tall fescue (*Lolium arundinaceum* Darbyshire ex. Schreb., KY-31) were naturally infected with *Epichloë coenophialum*[^80,81^], and uninfected (E⁻) seeds were acquired by eliminating the endophyte through the long-term storage of E⁺ seeds at room temperature. This procedure reduces the viability of the endophyte but not the seeds[^82^]. E⁺ and E⁻ seeds were originally obtained from...
Professor Keith Clay at Indiana University, USA. The seeds used in this experiment were several generations distant from the storage treatment and came from freely cross-pollinated field-grown parents. To re-isolate the endophyte, 30 E+ and 30 E− plant individuals were randomly sampled, and the method described by Latch & Christensen was used with a slight modification that the time for sodium hypochlorite treatment was 8–10 min, and the petri plates containing potato dextrose agar (PDA) were incubated in the dark at 25°C. Up to 4 weeks’ examination, only one species of endophyte, E. coenophialum, was isolated from E+ seedlings while no endophyte was found in E− seedlings. Meantime, seed germination rates for E+ and E− seeds were compared before the experiment. No significant differences were found between them, with regard to the number of days to first seedling emergence and germinations rates. Four weeks later, seven equally sized seedlings were transferred into each plastic pot (15 cm × 13.5 cm) filled with 1.4 kg of sterilized sand. After a week’s growth, they were differently treated and were placed into two separate growth chambers set at 400 or 800 ppm CO2. Plants were maintained at 30000 lux and a 12/12 h light/dark cycle at 25/20 °C, respectively. Endophyte status of the plants was checked both immediately before and after the experiment by microscopic examination from leaf sheaths stained with aniline blue described by Latch & Christensen. We found that seedlings from E+ seeds were all infected (100%) while no seedling from E− seeds was infected (0%).

Experiment design. The present study included two experiments. In the first experiment, we addressed the questions: does endophyte improve growth of the grass host under elevated CO2 concentration? If this is the case, how does nitrogen (N) availability affect the symbiosis-dependent benefits? In the second experiment, we addressed the question: does elevated CO2 affect pathogen resistance of grass-endophyte symbiont? From the first experiment, we found that endophyte-associated benefit only occurred in high N condition. So in the second experiment, test was performed only in high N level.

Experiment 1. A three factors randomized block design was used in this experiment. The first factor was two CO2 concentrations with two levels: ambient CO2 (400 ppm, AC) and elevated CO2 (800 ppm, EC). The second factor was N availability with two levels: high N (HN) and low N (LN). The third factor was endophyte infection status: endophyte-infected (E+) and uninfected (E−). Each treatment was replicated five times, totally 40 pots.

The nutrients were supplied by the addition of a modified Hoagland nutrient solution. The composition of the nutrient solution was 5.0 mM CaCl2, 5.0 mM KCl, 2.5 mM MgSO4·7H2O, 2.0 mM KH2PO4, 29 μM Na2-EDTA, 20 μM FeSO4·7H2O, 45 μM H3BO3, 6.6 μM MnSO4, 0.8 μM ZnSO4·7H2O, 0.6 μM H2MoO4, 0.4 μM CuSO4·5H2O, and pH 6.0 ± 0.1. Nitrogen was added in the form of NH4NO3, which was delivered as 1 mM N (LN) or 10 mM N (HN), respectively. During the experiment, 100 ml of nutrient solution was added once a week to each pot, a total of 9 times. Plants were watered as necessary with deionized water. In each block, the positions of the pots were randomly rotated each week to minimize location effects. The experiment lasted for 63 days.

Experiment 2. A three factors randomized block design was used in this experiment. The first factor was two CO2 concentrations with two levels: ambient CO2 (400 ppm, AC) and elevated CO2 (800 ppm, EC). The second factor was pathogen inoculation with two levels: uninoculated control (P−) and inoculated by Curvularia lunata (P+). The third factor was endophyte infection status: E+ and E−. Each treatment was replicated five times, totally 40 pots. Pathogen inoculation was performed after 8 weeks’ growing in the growth chamber with different CO2 concentrations. All treatments were sampled at the 6th day after pathogen inoculation.

Response variables in Experiment 1. Photosynthesis parameters. At the end of experiment 1, gas exchange measurements were made on the youngest fully expanded attached leaf in a pot with a LI-COR 6400 infrared gas analyzer (LI-Cor, Lincoln, NE, USA). Under 400 μmol mol−1 or 800 μmol mol−1 CO2, net photosynthetic rate (Pn) was measured at 1,500, 1,200, 1,000, 800, 600, 400, 200, 150, 100, 50, 30, 20 and 0 μmol m−2 s−1 PPFD (photosynthetic photon flux density). According to Pn-PPFD curve, Pmax were determined.
Growth and biomass. At the end of experiment 1, regular measurement of tiller number, leaf number, and shoot height of the longest tiller were made on all rameis. Then, the shoot and the root were harvested separately. The harvested material was ven-dried at 80 °C for biomass measurement and C and N analyses.

Carbon (C) and nitrogen (N) concentration. C and N concentrations were determined using the dry combustion method with an Elemental Analyser (Vario EL/micro cube, Elementar, Hanau, Germany).

Response variables in Experiment 2. Pathogen inoculation and lesion index recorded. C. lunata was obtained from Grassland Protection Institute, Lanzhou University, China. It was originally isolated from Poa pratensis. For inoculum, the pathogen was cultured on PDA at 25 °C for 2 weeks. Spores were washed with sterile distilled water and filtered through two-layer sterile gauze. A haemocytometer was used to count the spores, and the spore concentration was 13.44 × 10^{7}/mL. Plants were inoculated by spraying the spore suspensions using a sprayer until small droplets were seen on the leaves, and the control was sprayed with sterile distilled water. After inoculation, plants were immediately covered with a plastic bag for 36 h to maintain humidity.

Ten fully expanded mature leaves per pot were chosen for measuring the number and length of disease lesions. After measurement, pathogen spor concentration on the leaves was decided according to Nan & Li.

Soluble sugar, amino acid and lignin. Soluble sugar content was analyzed using the phenol-sulphuric acid method according to Buyssse and Mercx. Amino acids were analyzed by reverse-phase high-performance liquid chromatography (HPLC, Waters 1500-series) with pre-column derivatization using diinitrofluorobenzene (DNFB) according to Li and Sun. Lignin measurement was according to the procedure of Reddy, et al.

Statistical analyses. For the amino acids, we performed a principal components analysis (PCA) on the correlations among the 17 response variables and then performed factor rotation using the varimax method. After varimax rotation, we retained four rotated factors (RF). The RF variables and all other indexes were subjected to three-way analyses of variance (ANOVA). Differences between the means were compared using Duncan's multiple-range tests at P < 0.05. All statistical analyses were performed using SPSS 21.0 software.

References
1. IPCC. Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (ed. by T. F. Stocker, D. Qin, G. K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P. M. Midgley), pp. 148. Cambridge University Press, Cambridge, U. K. and New York, New York, doi:10.5061/dryad.56cn8015 10.1086/665032 (2013).
2. Ainsworth, E. A. & Long, S. P. What have we learned from 15 years of free-air CO2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO2. New Phytologist 165, 351–372 (2005).
3. Robinson, E. A., Ryan, G. D. & Newman, J. A. A meta-analytical review of the effects of elevated CO2 on plant–arthropod interactions highlights the importance of interacting environmental and biological variables. New Phytologist 194, 321–336 (2012).
4. Bryant, J. P., Chapin, F. S. & Klein, D. R. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos 40, 357–368 (1983).
5. Hu, S., Firestone, M. K. & Chapin, F. S. Soil microbial feedbacks to atmospheric CO2 enrichment. Trends in Ecology & Evolution 14, 433–437 (1999).
6. Zanetti, S. et al. Stimulation of symbiotic N2 fixation in Trifolium repens L. under elevated atmospheric CO2 in a grassland ecosystem. Plant Physiology 112, 575–583 (1996).
7. Johnson, N. C., Wolf, J. & Koch, G. Interactions among mycorrhizae, atmospheric CO2 and soil N impact plant community composition. Ecology Letters 6, 532–540 (2003).
8. Hunt, M. G., Rasmussen, S. P. C. D., Parsons, A. J. & Newman, J. A. Near-term impacts of elevated CO2, nitrogen and fungal endophyte-infection on Lolium perenne L. growth, chemical composition and alkaloid production. Plant Cell & Environment 28, 1345–1354 (2005).
9. Newman, J. A. et al. Effects of elevated CO2, nitrogen and fungal endophyte-infection on tall fescue: growth, photosynthesis, chemical composition and digestibility. Global Change Biology 9, 425–437 (2003).
10. Arnold, A. E. et al. Fungal endophytes limit pathogen damage in a tropical tree. Proceedings of the National Academy of Sciences of the United States of America 100, 15649–15654 (2003).
11. Clay, K. & Schardl, C. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. The American Naturalist 160(Suppl 4), S99–S127 (2002).
12. Schardl, C. L., Leuchtmann, A. & Spiering, M. J. Symbioses of grasses with seedborne fungal endophytes. Annual Review of Plant Biology 55, 315–340 (2004).
13. Clay, K. Fungal endophytes of grasses. Annual Review of Ecology & Systematics 21, 275–297 (1990).
14. Saikkonen, K., Wall, P., Helander, M. & Faeth, S. H. Evolution of endophyte–plant symbioses. Trends in Plant Science 9, 275–280 (2004).
15. Hesse, U. et al. Effects of Neotyphodium endophytes on growth, reproduction and drought-stress tolerance of three Lolium perenne L. genotypes. Grass & Forage Science 58, 407–415 (2003).
16. Burns, J. C. & Fisher, D. S. Intake and digestion of 'Jesup' tall fescue hay with a novel fungal endophyte, without an endophyte, or with a wild-type endophyte. Crop Science 46, 216–223 (2006).
17. Gibert, A. & Hazard, L. Endophyte infection of Festuca eskia enhances seedling survival to drought and cutting at the expense of clonal expansion. Journal of Plant Ecology 4, 201–208 (2011).
18. Ria, M. A., Mccueley, R. L. & Mitchell, C. E. Fungal endophyte infection and host genetic background jointly modulate host response to an aphid-transmitted viral pathogen. Journal of Ecology 101, 1007–1018 (2013).
19. Worchel, E. R., Giauque, H. E. & Kivlin, S. N. Fungal symbionts alter plant drought response. Microbial Ecology 65, 671–678 (2013).
20. Zurek, G., Wiesiórka, B. & Godzowski, D. Relations between bioclimatic variables and endophyte colonization of grasses in Poland. Fungal Ecology 6, 554–556 (2013).
21. Cheplick, G. P., Clay, K. & Marks, S. Interactions between infection by endophytic fungi and nutrient limitation in the grasses Lolium perenne and Festuca arundinacea. New Phytologist 111, 89–97 (1989).
22. Lewis, G. C., Bakken, A. K., Macduff, J. H. & Raisstrup, N. Effect of infection by the endophytic fungus Acremonium lolii on growth and nitrogen uptake by perennial ryegrass (Lolium perenne) in flowing solution culture. Annals of Applied Biology 129, 451–460 (1996).
63. Guo, H. et al. Elevated CO₂ decreases the response of the ethylene signaling pathway in Medicago truncatula and increases the abundance of the pea aphid. New Phytologist 201, 279–291 (2014).

64. Johnson, S. N., Ryalls, J. M. W. & Karley, A. J. Global climate change and crop resistance to aphids: contrasting responses of lucerne genotypes to elevated atmospheric carbon dioxide. Annals of Applied Biology 165, 62–72 (2014).

65. Nowak, H. & Komor, E. How aphids decide what is good for them: experiments to test aphid feeding behaviour on Tanacetum vulgare (L.) using different nitrogen regimes. Oecologia 163, 973–984 (2010).

66. Belesky, D. P. & Fedders, J. M. Does endophyte influence regrowth of tall fescue? Journal of Plant Pathology 92, 357–358 (2015).

67. Sullivan, T. J. et al. Symbiotic-mediated changes in Lolium arundinaceum inducible defenses: evidence from changes in gene expression and leaf composition. New Phytologist 176, 673–679 (2007).

68. Ma, M., Christensen, M. J. & Nan, Z. Effects of the endophyte Epichloë festucae var. lolii of perennial ryegrass (Lolium perenne) on indicators of oxidative stress from pathogenic fungi during seed germination and seedling growth. European Journal of Plant Pathology 141, 571–583 (2015).

69. Pangga, I. B., Chakraborty, S. & Yates, D. Canopy size and induced resistance in Stylosanthes scabra determine Anthracnose severity at high CO₂. Phytomorphology 94, 221–227 (2004).

70. Plessl, M., Elstner, E. F., Renningen, H., Habermeyer, J. & Heiser, I. Influence of elevated CO₂ and ozone concentrations on late blight resistance and growth of potato plants. Environmental & Experimental Botany 60, 447–457 (2007).

71. Percy, K. E. et al. Altered performance of forest pests under atmospheres enriched by CO₂ and O₃. Nature 420, 403–407 (2002).

72. Pugliese, M., Liu, J., Tittone, P., Garibaldi, A. & Gullino, M. L. Effects of elevated CO₂ and temperature on interactions of zucchini powdery mildew. Phytopathologia Mediterranea 51, 480–487 (2012).

73. Mitchell, C. E., Reich, P. B., Tilman, D. & Groth, J. V. Effects of elevated CO₂, nitrogen deposition, and decreased species diversity on foliar fungal plant disease. Global Change Biology 9, 438–451 (2003).

74. Thompson, G. B. & Drake, B. G. Insects and fungi on a C₃ sedge and a C₄ grass exposed to elevated atmospheric CO₂ concentrations in open-top chambers in the field. Plant Cell & Environment 17, 1161–1167 (1994).

75. McIrvine, A., Reid, C., Ka, Hart, E. & Jackson, R. Elevated CO₂ reduces disease incidence and severity of a red maple fungal pathogen via changes in host physiology and leaf chemistry. Global Change Biology 11, 1828–1836 (2005).

76. Hammond-Kosack, K. E. & Jones, J. D. Resistance gene-dependent plant defense responses. Plant Cell 8, 1773–1791 (1996).

77. Funnell-Harris, D., Jeffreys, P. & Scottie, S. Alteration in lignin biosynthesis restricts growth of Fusarium spp. in brown midrib sorghum. Phytopathology 100, 671–681 (2010).

78. Qawasmeh, A., Obied, H. K., Raman, A. & Wheatley, W. Influence of fungal endophyte infection on phenolic content and antioxidant activity in grasses: interaction between Lolium perenne and different strains of Neotyphodium lolii. Journal of Agricultural and Food Chemistry 60, 3381–3388 (2012).

79. Omacini, M., Chanetion, E. J. & Ghera, C. M. A framework for understanding the ecosystem consequences of endophyte grass symbioses. Neotyphodium in Cool-Season Grasses: Current Research and Applications (eds C. Roberts, C. P. West & D. Spiers), pp. 141–161. Blackwell Scientific, Oxford, UK, doi:10.1111/mec.12625 (2005).

80. Morganjones, G. & Gams, W. Notes on Hypomyces. XLI. An endophyte of Festuca arundinacea and the anamorph of Epichloë typhina, new taxa in one of two new sections of Acremonium. Mycotaxon 13, 113–117 (1982).

81. Leuchtmann, A., Bacon, C. W., Schardl, C. L., White, J. F. & Tadych, M. Nomenclatural realignment of Neotyphodium species with Neotyphodium genus. Mycologia 106, 202–215 (2014).

82. Clay, K. & Holah, J. Fungal endophyte symbiosis and plant diversity in successional fields. Science 285, 1742–1745 (1999).

83. Latch, G. C. M. & Christensen, M. J. Artificial infection of grasses with endophytes. Annals of Applied Biology 107, 17–24 (1985).

84. Nan, Z. B. & Li, C. J. Neotyphodium in native grasses in China and observations on endophyte / host interactions. Proceedings of the 4th international Neotyphodium-grass interactions symposium, 41–50, doi:10.1111/mec.12651 (2000).

85. Buesse, J. & Merckx, R. An improved colorimetric method to quantify sugar content of plant tissue. Journal of Experimental Botany 44, 1627–1629 (1993).

86. Li, D. & Sun, J. Determination of 18 kinds of amevo acids by HPLC with precolumn 2,4-dinitrofluorobenzene derivatization. Chemical Analysis & Meterage 13, 18–20 (2004).

87. Reddy, M. V. R., Arul, J., Angers, P. & Couture, L. Chitosan treatment of wheat seeds induces resistance to Fusarium graminearum and improves seed quality. Journal of Agricultural & Food Chemistry 47, 1208–1216 (1999).

88. Rasmussen, S., Parsons, A. J., Fraser, K., Xue, H. & Newman, J. A. Metabolic profiles of Lolium perenne are differentially affected by genotypes to elevated atmospheric carbon dioxide. Annals of Applied Biology 165, 62–72 (2014).

Acknowledgements

This research was funded by National Key Research and Development Program (2016YFC0500702) and National Natural Science Foundation (31570433) of China.

Author Contributions

A.R. conceived and designed the experiments. W.C., H.L. and Wurihan performed the experiments. W.C. and A.R. analyzed the data. Y.G. contributed reagents, materials and analysis tools. The manuscript was written by A.R. and S.C., and reviewed by all authors.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017