Global Change Biology (2007) **13,** 1238–1249, doi: 10.1111/j.1365-2486.2007.01347.x

Plant nitrogen acquisition and interactions under elevated carbon dioxide: impact of endophytes and mycorrhizae

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Abstract

Both endophytic and mycorrhizal fungi interact with plants to form symbiosis in which the fungal partners rely on, and sometimes compete for, carbon (C) sources from their hosts. Changes in photosynthesis in host plants caused by atmospheric carbon dioxide (CO_2) enrichment may, therefore, influence those mutualistic interactions, potentially modifying plant nutrient acquisition and interactions with other coexisting plant species. However, few studies have so far examined the interactive controls of endophytes and mycorrhizae over plant responses to atmospheric CO_2 enrichment. Using *Festuca arundinacea* Schreb and *Plantago lanceolata* L. as model plants, we examined the effects of elevated CO_2 on mycorrhizae and endophyte (*Neotyphodium coenophialum)* and plant nitrogen (N) acquisition in two microcosm experiments, and determined whether and how mycorrhizae and endophytes mediate interactions between their host plant species. Endophyte-free and endophyte-infected *F. arundinacea* varieties, *P. lanceolata* L., and their combination with or without mycorrhizal inocula were grown under ambient (400 μmol mol^{-1}) and elevated CO_2 (ambient + 330 μmol mol^{-1}). A ^{15}N isotope tracer was used to quantify the mycorrhiza-mediated plant acquisition of N from soil. Elevated CO_2 stimulated the growth of *P. lanceolata* greater than *F. arundinacea*, increasing the shoot biomass ratio of *P. lanceolata* to *F. arundinacea* in all the mixtures. Elevated CO_2 also increased mycorrhizal root colonization of *P. lanceolata*, but had no impact on that of *F. arundinacea*. Mycorrhizae increased the shoot biomass ratio of *P. lanceolata* to *F. arundinacea* under elevated CO_2. In the absence of endophytes, both elevated CO_2 and mycorrhizae enhanced ^{15}N and total N uptake of *P. lanceolata* but had either no or even negative effects on N acquisition of *F. arundinacea*, altering N distribution between these two species in the mixture. The presence of endophytes in *F. arundinacea*, however, reduced the CO_2 effect on N acquisition in *P. lanceolata*, although it did not affect growth responses of their host plants to elevated CO_2. These results suggest that mycorrhizal fungi and endophytes might interactively affect the responses of their host plants and their coexisting species to elevated CO_2.

Keywords: elevated CO_2, endophyte, *Festuca arundinacea*, mycorrhizae, ^{15}N tracer, plant N acquisition, *Plantago lanceolata*

Received 6 March 2006; revised version received 20 September 2006 and accepted 28 November 2006

Introduction

Carbon dioxide (CO_2) enrichment in the atmosphere in general stimulates photosynthetic activity and growth of C_3 plants. This may in turn alter the availability of photosynthates for plant-associated microbes (Hu *et al*., 1999), modifying plant–microbial interactions such as

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plant–rhizobial symbiosis (Zanetti *et al*., 1996), mycorrhizae (Johnson *et al*., 2003), and plant–endophyte complexes (Newman *et al*., 2003; Hunt *et al*., 2005).

More than 80% of the higher plant species examined are associated with arbuscular mycorrhizal (AM) fungi (Smith & Read, 1997). Many studies have shown that elevated CO_2 often increases mycorrhizal colonization of roots and to a lesser degree mycorrhizal activities (Rillig *et al*., 1999; Hartwig *et al*., 2002; Treseder, 2004; Hu *et al*., 2005). This CO_2-enhancement may alter plant nutrient uptake and plant interactions with their neighbors (van der Heijden *et al*., 1998; Smith *et al*., 1999; O'Connor *et al*., 2002), particularly if mycorrhizae in the coexisting species have differential responses to elevated CO_2.

Similar to mycorrhizal fungi, endophytic fungi within asymptomatic aerial tissues of plants represent a ubiquitous component of terrestrial plant communities (Arnold *et al*., 2003). About two-thirds of cold-season grasses such as tall fescue (*Festuca arundinacea* Schreb) and ryegrass (*Lolium perenne*) form symbionts in their aboveground parts with endophytic fungi (Müller & Krauss, 2005). For example, tall fescue is often associated with a systemic fungal endophyte, *Neotyphodium coenophialum* Morgan-Jones and Gams (Ascomycota: Clavicipitaceae) (Bacon *et al*., 1977; Glenn *et al*., 1996). Endophytes often enhance the resistance of the host plants to environmental stresses such as drought (Richardson *et al*., 1993; Schardl *et al*., 2004) and pathogens (Arnold *et al*., 2003). Endophytic fungi in tall fescue produce a group of toxic compounds named ergot alkaloids (Hill *et al*., 1994) that protect host plants from herbivores (Bree, 1994; Clay & Schardl, 2002), fungi (Latch, 1993) and nematodes (Gwinn & Bernard, 1993) and increase host resistance to both water and nitrogen (N) stress (Lyons *et al*., 1990; Elmi & West, 1995; Clay & Schardl, 2002), resulting in increased host tolerance to marginal soil environments.

Both mycorrhizal and endophytic fungi solely rely on C sources from their host plants. In some plants, endophytic and mycorrhizal fungi may compete for photosynthates (Vicari *et al*., 2002). Therefore, factors that alter plant photosynthesis and subsequent allocation of photosynthetic products may profoundly influence the colonization of those mutualistic fungi, as well as their functioning. Because the CO_2 concentration is a limiting factor for many C_3 plants, the rising CO_2 concentration in the atmosphere can enhance the availability of photosynthates for both mycorrhizal (Staddon & Fitter, 1998; Johnson *et al*., 2003; Treseder, 2004) and endophytic fungi (Marks & Clay, 1990). Over the last decade, many experiments have examined the effect of elevated CO_2 on mycorrhizae (see recent meta-analysis by Treseder, 2004) and the subsequent effects on host growth. However, only a few experiments have explicitly investigated the role of endophytic fungi in mediating the response of their host grasses to elevated CO_2 (Marks & Clay, 1990; Marks & Lincoln, 1996; Applebee *et al*., 1999; Newman *et al*., 2003; Hunt *et al*., 2005). Among over 50 publications on perennial ryegrass, only two examined the status of endophytic fungi (Marks & Lincoln, 1996; Hunt *et al*., 2005). In tall fescue, Newman *et al*. (2003) examined the response of endophyte-infected (E+) and endophyte-free (E−) plants to elevated CO_2 and found that the presence of endophytes modify plant response by altering N metabolism. In spite of common existence of both mycorrhizae and endophytes in cold-season grasses, to our knowledge, no experiments have explicitly examined any potential impacts of elevated CO_2 on the interaction between endophytes and mycorrhizae.

In the present study, we examined how elevated CO_2 affects endophytes and mycorrhizae, and their functions, using tall fescue and *Plantago lanceolata* as model species. Tall fescue is one of the most important perennial grasses, occupying more than 35 million ha as a forage, turf or soil conservation plant in the United States alone (Ball *et al*., 1993; Rudgers *et al*., 2004). Because of high tolerance to environmental stresses and adaptation to various climatic conditions, tall fescue is a common species in many natural ecosystems and is an invasive species to some endangered habitats across the world (Clay & Holah, 1999; Saikkonen, 2000; Rudgers *et al*., 2004). In general, tall fescue is not readily colonized by mycorrhizae and the cause for low mycorrhizal infection is not exactly known. It was suggested that endophytes compete with mycorrhizae for carbon resources within the host or that endophytes are inhibiting AM colonization through fungitoxic compounds (Chu-Chou *et al*., 1992; Guo *et al*., 1992; Matthews & Clay, 2001; Müller, 2003). A few recent studies also showed that *F. arundinacea* is very responsive to elevated CO_2 (Newman *et al*., 2003), but whether CO_2-enhancement of C availability will alter mycorrhizal infection and activities in fescue roots remains unexplored. Tall fescue coexists with many cool-season grasses and forbs in pastures and natural habitats. Plantago (*P. lanceolata* L.) is a common weedy species in many pastures and natural habitats dominated by tall fescue across the world (Barton & Nestor, 1979). It is highly mycorrhizal (Staddon *et al*., 1999; Hodge *et al*., 2001) and is very responsive to CO_2 enrichment (Rouhier & Read, 1998). An understanding of CO_2 effects on mycorrhizae and endophytes will provide insight into the role of those symbiotic fungi on resource acquisition/partitioning and interactions among coexisting host plant species under anticipated CO_2 scenarios.

We conducted two experiments investigating how mycorrhizae and endophytes mediate the response of tall fescue and *P. lanceolata* to elevated CO_2 both in monoculture and mixture. The specific objectives of the experiment were to examine (1) whether endophytes affect mycorrhizal responses to elevated CO_2 in *F. arundinacea* and *P. lanceolata*, and (2) whether and how endophytes and mycorrhizae mediate nutrient acquisition and interactions of their host plants under elevated CO_2.

Materials and methods

The CO_2 facility

A microcosm study was conducted in the CO_2 exposure facility at the USDA Air-Quality greenhouse at North Carolina State University. The CO_2 facility consists of a 9 m × 12 m greenhouse bay containing 20 continuously stirred tank reactor (CSTR) chambers each measuring 1.2 m diameter × 1.4 m tall for exposure of plants to CO_2 gas (Booker *et al*., 2000). The chambers were made of transparent materials that allowed full light penetration. Dispensing and monitoring of gas treatments are accomplished in a laboratory adjacent to the greenhouse. A blower system provided a constant flow of charcoal filtered air through each CSTR. For those chambers assigned to an elevated CO_2 treatment, compressed CO_2 was added to the air entering the CSTR using a rotamenter to control flow so that CO_2 concentration was maintained at target level. The air continuously moved out the CSTR and thus alleviated the heating effect of chambers. Monitoring of CO_2 concentration was accomplished using computer-activated solenoid valves to direct gas exiting the CSTR into infrared analyzers (model 6252, LiCor Inc., Lincoln, NE, USA). The computer collected and averaged temperature and CO_2 data for analysis.

Experiment 1: effects of mycorrhizae on F. arundinacea *and* P. lanceolata *responses to elevated CO_2 in monoculture and mixture*

Plant seeds and mycorrhizal fungal inocula. Seeds of endophyte-free tall fescue (cv. Kentucky-31) (E−K) and *P. lanceolata* (PL) were obtained from commercial suppliers (The Wyatt-Quarles Seed Co., Raleigh, NC, USA, and The Nurseries, Billingbear Park, Wokingham, Berkshire, UK, respectively). Mycorrhizal inoculum was obtained from a field at the Center for Environmental Farming Systems, North Carolina State University, near Goldsboro, NC, USA. The AM inoculum was a mixture of soil with spores, hyphae and root residues containing *Glomus etunicatum*, *Glomus clarum*, *Gigaspora margarita*, *Gigaspora rosea*, *Scutellospora heterogama*, *Scutellospora pellucida*, *Acaulospora scrobiculata*, *Acaulospora laevis*, *Acaulospora koskei*, *Acaulospora mellea* and *Acaulospora* sp.

Experimental design and treatments. The experiment was established in a split–split plot design with atmospheric CO_2 concentrations (ambient and elevated CO_2) as the mainplot factor, mycorrhizae (mycorrhizae and nonmycorrhizae) as subplot factors, and plants (E−K, PL and their mixture) as sub-subplot factors. Six CSTR chambers were blocked into three blocks (i.e. three chambers per CO_2 level). Either an ambient (400 μmol mol^{-1}) or elevated CO_2 level (ambient-+330 μmol mol^{-1}) was randomly assigned to each chamber within a block. The elevated CO_2 concentration corresponds approximately to the predicted CO_2 concentration by the end of this century (IPCC, 2001). Two microcosm units (one with mycorrhizae and another with no mycorrhizae) were placed in each chamber.

A microcosm unit containing six 2.7 L compartments (described in detail by Tu *et al*., 2006) was employed with three compartments on one side designated as Host and the other three as Test compartments. Each Host/Test pair in a microcosm was separated by a replaceable mesh that allowed penetration by hyphae but not roots. The soil used was a mixture of one part of a sandy loam soil with two parts of pure sand (by weight). The mixture had an extractable N of 7.6 mg N kg^{-1} and a pH of 6.7. Each compartment of the microcosm was filled with 3.0 kg of the sterilized soil mixture. For mycorrhizal microcosms, 10.0 g AM inoculum was incorporated into the soil in each Host compartment. The nonmycorrhizal microcosms received 100 mL of microbial filtrate solution filtrated by Whatman No. 1 filter paper from 10.0 g AM inoculum (with no mycorrhizal spores) and the sterilized residues of the inoculum to correct for possible differences in the microbial community and nutrients between mycorrhizal and nonmycorrhizal treatments.

Seeds of *P. lanceolata* (20 seeds), *F. arundinacea* (E−K, 40 seeds) and their mixture (10 and 20 seeds, respectively) were respectively sown in the three Host compartments in each microcosm. After emergence, E−K and PL seedlings were thinned to ensure the same number of plants in each compartment. The plants were grown in ambient light and temperature conditions. The plants were watered with distilled water daily. The TEST compartments were also watered but less frequently in order to maintain the soil moisture so that AM fungal hyphae could grow into the Test soils. Eight weeks after seeding, mineral N (NH_4NO_3) was added at a rate of 10 mg N kg^{-1} soil to each Host and Test compartment.

^{15}N tracer. To quantify mycorrhizally mediated plant N uptake, the ^{15}N tracer was injected uniformly as ^{15}N-enriched mineral N ($(NH_4)_2SO_4$, 99.7% atom ^{15}N) in DI water at a rate of 3.0 mg N kg^{-1} soil into each Test compartment 1 week before harvesting.

Collection and analyses of plant and soil samples. The plants were harvested 14 weeks after seeding. Root systems were separated from shoots and the fresh root biomass was weighed immediately. Roots of the two species in the mixture were also visually separated. Half of each fresh root sample was stored at 4 °C for quantification of AM fungal colonization and the remaining half was oven dried (65 °C for 48 h) and used for biomass measurement. Soil samples in the Host and Test compartments were collected separately and stored at 4 °C for extractable N quantification.

The fresh shoot biomass was weighed immediately following the harvest. Ten tillers from each sample were stored at −20 °C until they were tested for the presence of endophytes. The remaining shoots were dried at 65 °C for 48 h and weighed, and the total shoot biomass was obtained by adding the dry weight of those10 tillers later. The oven-dried shoots were used for measurements of total N and ^{15}N. Root biomass C was based on ash-free dry weight because of potential soil contamination. The C concentration of roots was analyzed on a Perkin-Elmer 2400 CHNS/O elemental analyzer (Norwalk, CT, USA). Root biomass C was calculated by multiplying the C concentration of roots and the total weight of roots.

Experiment 2: Endophytic effects on responses of F. arundinacea *and* P. lanceolata *to elevated CO_2 in mixture*

Seeds and mycorrhizal fungal inocula. Seeds of *P. lanceolata* were from the same seedlot as was used in Experiment 1. Three varieties of *F. arundinacea* [e.g. endophyte-free cv. Jesup (E−J), endophyte-infected cv. Jesup (E + J) and endophyte-infected cv. Kentucky-31 (E + K)], were used to examine endophyte effects on responses to elevated CO_2 of both *P. lanceolata* and *F. arundinacea* growing in mixture. The E−J and E + J seeds were provided by Pennington Seed Inc. (Madison, GA, USA), and the E + K seeds were purchased from the Wyatt-Quarles Seed Co. While cultivar E−J was the endophyte-free isogenic line of cultivar (E + J), E + K was the same variety of E−K in Experiment 1 except for the endophyte status. Mycorrhizal inoculum was a mixture of soil with spores, hyphae and root residues containing *Glomus intraradices*, *G. margarita* and *S. heterogama*, which had the same original source as that in the Experiment 1.

Experimental design and treatments. The experiment was a split-plot design with atmospheric CO_2 concentrations (ambient and elevated CO_2) as the mainplot factor, and endophyte/variety (E−J, E + J and E + K) as subplot factors. Either ambient (400 μmol mol^{-1}) or elevated CO_2 level (ambient + 330 μmol mol^{-1}) was randomly assigned to four pairs of chambers (i.e. four replicates per CO_2 level). Each compartment of the microcosm units was filled with 3.0 kg of the sterilized soil mixture and then the Host compartment inoculated with 10.0 g of mycorrhizal inocula. Two microcosm units were placed in each chamber to provide two levels of additional N inputs (10 and 35 mg N kg^{-1} soil) in which mineral N (NH_4NO_3) in solution was added to both Test and Host compartments.

Seeds of *P. lanceolata* (10 seeds) and *F. arundinacea* (20 seeds) of E−J, E + J or E + K were sown into each Host compartment. Seedlings of *P. lanceolata* and *F. arundinacea* plants in each compartment were then thinned to ensure each compartment have exactly same numbers of plants. The plants were managed similarly as in Experiment 1 and this experiment was terminated 18 weeks after seeding. Plant samples were collected for biomass and analyses.

^{15}N tracer. The ^{15}N at a rate of 3.0 mg N kg^{-1} soil in form of $(NH_4)_2SO_4$ (99.7% atom ^{15}N) solution was injected uniformly in each Test compartment 3 weeks before harvesting to quantify mycorrhizally mediated plant N uptake.

Measurements of ^{15}N and N in soil and plant biomass

A subsample (ca. 1.0 g) of each dried shoot sample was ground using an 8000D SPEX CertiPrep Dual Mixer (SPEX CertiPrep, Metuchen, NJ, USA). Shoot N concentrations and N isotope ratios (^{14}N and ^{15}N) were determined using a ThermoFinnigan DELTAPlus continuous flow isotope ratio mass spectrometer (CF-IRMS, Thermo Finnigan DELTA Plus, Waltham, MA, USA). Sample ^{15}N concentrations were calculated from fractional abundance [$^{15}N/(^{14}N + ^{15}N)$] and total N content (Hu *et al.*, 2001). The amount of plant ^{15}N uptake mediated by mycorrhizae was calculated by ^{15}N in mycorrhizal samples minus ^{15}N in nonmycorrhizal samples. Ammonium (NH_4^+) and nitrate (NO_3^-) in the soil were extracted using 0.5 M K_2SO_4 and their concentrations were determined using a fluid injection autoanalyzer (Lachat Instruments, Milwaukee, WI, USA).

Mycorrhizal colonization of plant roots

Root colonization by mycorrhizal fungi was quantified using a dissection microscope (× 20–40) after cleaning

the roots in 10% KOH (w/v) and staining with trypan blue in an acid glycerol solution. A variation of the gridline intersection method, developed by Giovannetti & Mosse (1980), was used to determine the percentage of root length in which arbuscles, vesicles or hyphae occurred.

Presence of endophytes in tall fescue seeds and plants

The presence of endophyte in both tillers and seeds was determined by a histological staining method (Hiatt *et al.*, 1999). Before the seeds were planted, 100 tall fescue seeds were soaked in a 5% NaOH solution at room temperature for 12–16 h. The seeds were rinsed thoroughly in running tap water and were then stained with aniline blue-lactic acid solution. The stained seeds were crushed slightly on slides and examined under a compound microscope. Plants collected at the final harvest were further examined for the existence of endophytes. The longitudinal sections from each frozen tiller sheath were stained with the aniline blue-lactic acid solution and then examined using a compound microscope.

Statistical analysis

Data from each experiment were subjected to analysis of variance using the general linear model procedure of the STATISTICAL ANALYSIS SYSTEM package (v. 8.02, SAS Institute, Cary, NC, USA) with appropriate error term to either a split–split plot (Experiment 1) or a split plot (Experiment 2) design. The significant differences were determined at the 95% probability level.

Results

Experiment 1: effects of mycorrhizae on responses of F. arundinacea *and* P. lanceolata *to elevated CO_2 in monoculture and mixture*

Shoot biomass, root biomass and shoot biomass ratio (PL : FA) of P. lanceolata *and* F. arundinacea. Elevated CO_2 significantly increased shoot biomass, but mycorrhizae did not influence shoot biomass (Fig. 1a, $P > 0.68$). In monoculture, elevated CO_2 enhanced shoot biomass of both *F. arundinacea* and *P. lanceolata* with larger effects on *P. lanceolata* (with a 52.0% increase) than on *F. arundinacea* (with a 30.4% increase) (Fig. 1a), regardless of the mycorrhizal status. In the mixture, total shoot biomass increased significantly under elevated CO_2 (Fig. 1a), and no significant mycorrhizal effect was observed (Fig. 1a). Both elevated CO_2 and mycorrhizae significantly increased the PL : FA ratio of shoot biomass in the mixture (Fig. 2a). Also, root biomass C was significantly increased by elevated CO_2 ($P < 0.02$) (Fig. 1b).

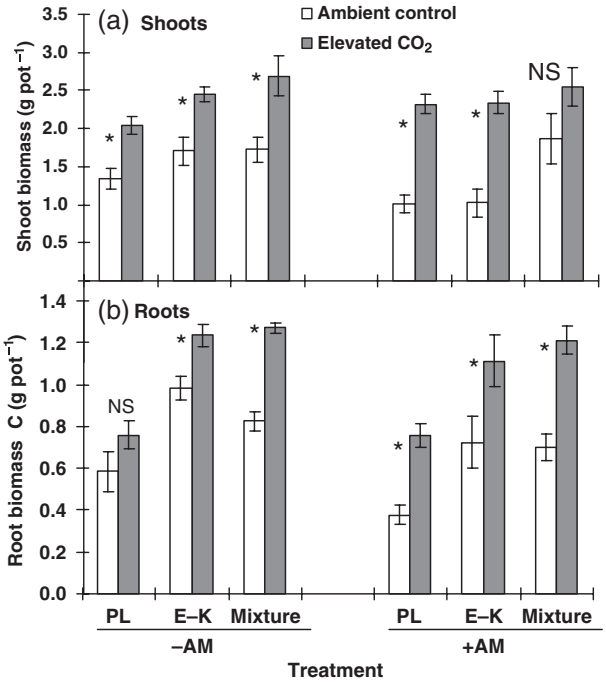

Fig. 1 Plant biomass as influenced by elevated carbon dioxide (CO_2) and arbuscular mycorrhizae (AM). (a) Shoot biomass and (b) root biomass in Experiment 1. Bars depict treatment means and standard errors (SE). *$P \leq 0.05$; NS, not significant between ambient and elevated CO_2; PL, *Plantago lanceolata* alone; E−K, *F. arundinacea*, endophyte-free cv. Kentucky-31 alone; Mixture, PL and E−K together.

Shoot biomass N and biomass ^{15}N. Elevated CO_2 did not significantly affect total shoot biomass N ($P > 0.53$), but AM fungi and plant species significantly affected shoot biomass N ($P < 0.05$ and $P < 0.001$, respectively; Fig. 3). Mycorrhizae significantly enhanced shoot biomass N of *P. lanceolata*, altering N distribution between *P. lanceolata* and *F. arundinacea* in the mixture. Under the ambient CO_2, biomass N in *P. lanceolata* shoots only accounted for 13.7% and 17.5% of total biomass N in the absence and presence of mycorrhizae, respectively. However, under elevated CO_2, this number increased to 26.9% and 40.3%, respectively (Fig. 3).

Mycorrhizae significantly increased ^{15}N uptake of *P. lanceolata* but not of *F. arundinacea* under both CO_2 concentrations. This mycorrhizal effect was also significantly affected by CO_2 concentrations (Fig. 4). Compared with the ambient CO_2, elevated CO_2 significantly increased ^{15}N uptake of *P. lanceolata* both in the monoculture and in the mixture, but had no effect on shoot ^{15}N in *F. arundinacea* (Fig. 4). Because *P. lanceolata* only consisted of a small proportion of the

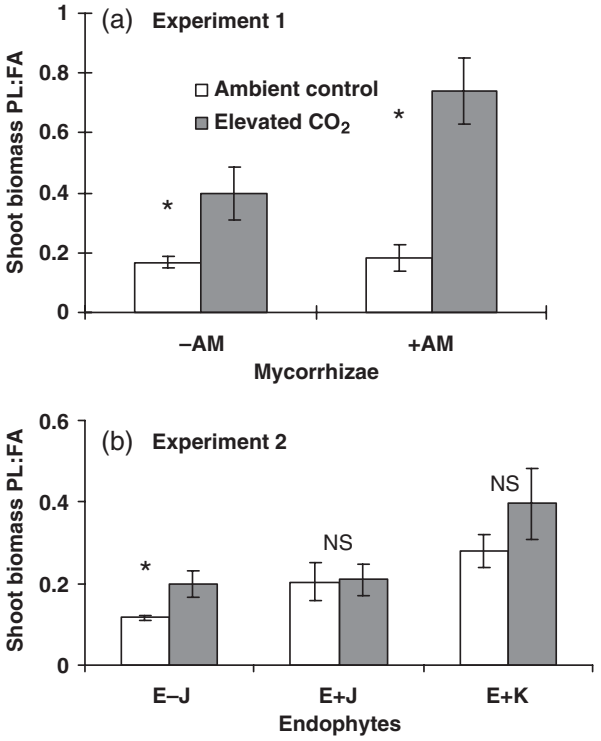

Fig. 2 Effects of arbuscular mycorrhizae (AM) (a) or endophytes (b) on the PL : FA ratio of shoot biomass in mixture under elevated carbon dioxide (CO_2). PL, *Plantago lanceolata*; FA, *Festuca arundinacea*; E−J, endophyte-free cv. Jesup; E+J, endophyte-infected cv. Jesup; E+K, endophyte-infected cv. Kentucky-31. Bars depict treatment means and standard errors (SE). $^*P \leq 0.05$ between ambient and elevated CO_2.

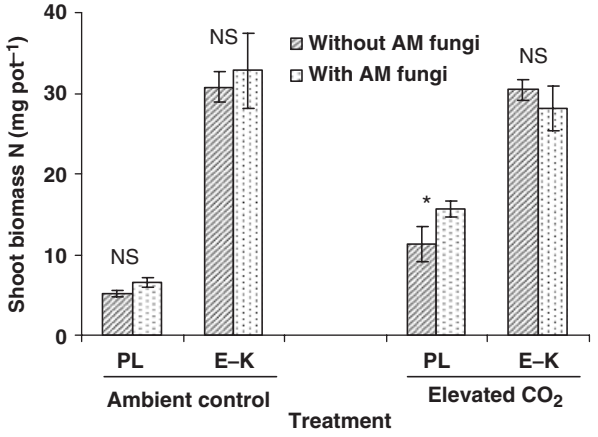

Fig. 3 Elevated carbon dioxide (CO_2) and arbuscular mycorrhiza effects on nitrogen (N) distribution between *Plantago lanceolata* (PL) and *Festuca arundinacea* (E−K) in their mixture in the Experiment 1. Bars depict treatment means and standard errors (SE). $^*P \leq 0.05$.

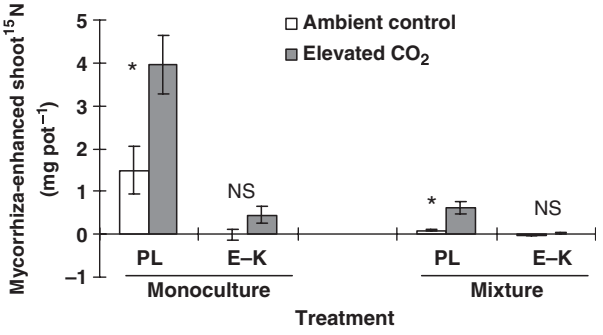

Fig. 4 Mycorrhiza-mediated ^{15}N acquisition of *Plantago lanceolata* and *Festuca arundinacea* in monoculture or mixture under ambient and elevated carbon dioxide (CO_2) in the Experiment 1. Shoot ^{15}N values refer to the ^{15}N excess in shoot biomass compared with the corresponding no-mycorrhizae control and were obtained by subtracting shoot ^{15}N content in the no-mycorrhizae control from the mycorrhizal treatment. Bars depict treatment means and standard errors (SE). $^*P \leq 0.05$; NS, not significant between ambient and elevated CO_2. Abbreviations refer to Fig. 1.

total biomass, mycorrhizal enhancement of total shoot ^{15}N content was reduced in the mixture (Fig. 4).

Soil extractable N in Host and Test compartments. No significant differences in soil extractable N in the Host soils were found among all treatments (data not shown), with the average of soil extractable N being at about 2.0 mg N kg^{-1} soil. However, soil extractable N in the Test soils was reduced by mycorrhizae, with significant reduction only in soils with *F. arundinacea* under ambient CO_2 and with *P. lanceolata* under elevated CO_2 ($P < 0.05$, Fig. 5). Compared with their respective ambient controls, elevated CO_2 tended to reduce extractable N in the Test soils in all treatments (Fig. 5), but this effect was not statistically significant ($P = 0.21$).

Mycorrhizal colonization of plant roots and the presence of endophyte in tillers and seeds. No obvious mycorrhizal colonization was found in the roots of both plant species in the no-mycorrhizal treatments under either CO_2 levels. When inoculated with mycorrhizae, mycorrhizal root colonization of *F. arundinacea* was still very low (<5%) both in monoculture and in mixture, regardless of atmospheric CO_2 concentrations (data not shown). In contrast, mycorrhizal root colonization of *P. lanceolata* was 31.0% in monoculture and 27.0% in mixture under ambient CO_2, and, respectively, increased to 46.0% and 37.0% under elevated CO_2 (Fig. 6a). Total root length colonized by mycorrhizal fungi tended to increase under elevated CO_2 but this effect was not statistically significant ($P = 0.24$). As to the endophytic status of *F. arundinacea*, neither the seeds nor the tillers collected at the end of the experiment were infected with endophytes.

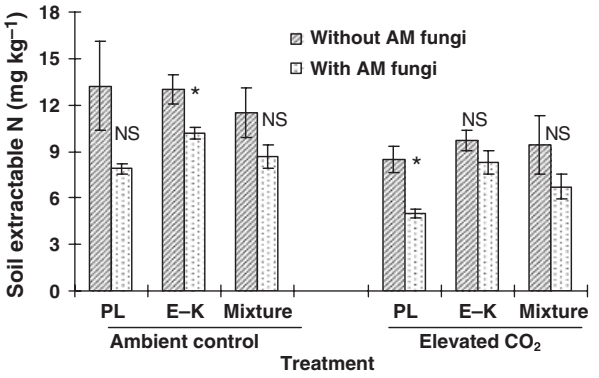

Fig. 5 Soil extractable nitrogen (N) in the Test compartments as affected by elevated CO_2 and arbuscular mycorrhizae (AM) in Experiment 1. Bars depict treatment means and standard errors (SE). *$P \leq 0.05$; NS, not significant between mycorrhizae (+AM) and no-mycorrhizae (−AM). Abbreviations refer to Fig. 1.

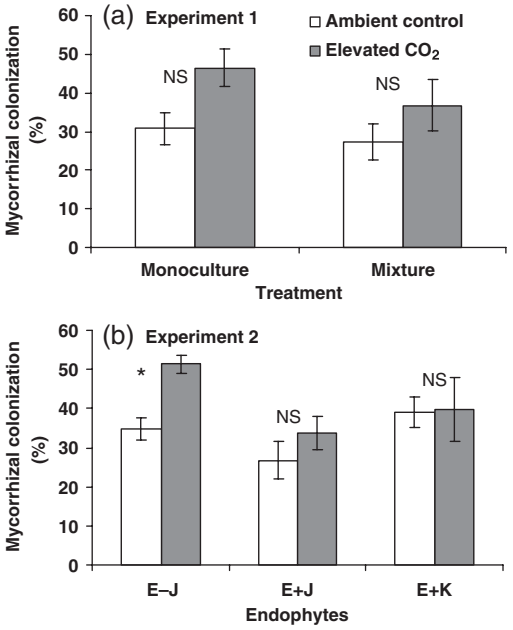

Fig. 6 Effects of elevated CO_2 on mycorrhizal root colonization (MRC) of *Plantago lanceolata* both in monoculture and in mixture with different fescue varieties. (a) Monoculture and mixture with endophyte-free fescue plants in Experiment 1. (b) Mixture with fescue varieties of different endophytic status in Experiment 2. Bars depict treatment means and standard errors (SE). *$P \leq 0.05$; NS, not significant between ambient and elevated CO_2.

Experiment 2: endophytic effects on responses of F. arundinacea *and* P. lanceolata *to elevated CO_2 in plant mixtures*

Shoot biomass, root biomass and shoot biomass ratio (PL : FA) of P. lanceolata *and* F. arundinacea *in the mixture.* Endophytes influenced shoot biomass of *P. lanceolata* ($P < 0.01$), but not *F. arundinacea* ($P = 0.9$) (Fig. 7a). Compared with the E−control (PL∼E−J), shoot biomass of *P. lanceolata* under both ambient and elevated CO_2 was significantly higher in the PL∼E+J and PL∼E+K mixtures with an average increase of 23% and 90%, respectively. This led to a significant increase in the shoot biomass ratios of PL : FA in the presence of endophytes (Fig. 2b, $P < 0.003$). Elevated CO_2 significantly increased shoot biomass of *P. lanceolata* in the PL∼E−J combination but had no significant effects on *F. arundinacea* shoot biomass (Fig. 7a), increasing the PL : FA biomass ratios in the PL∼E−J combination (Fig. 2b). However, this CO_2 effect was offset by endophytes as the PL : FA ratios were not affected by CO_2 enrichment in the PL∼E+J and PL∼E+K mixtures (Fig. 2b). Also, CO_2 enrichment enhanced root biomass of *F. arundinacea* but not *P. lanceolata* (Fig. 7b). The presence of endophytes reduced root biomass of host fescue plants by 8–10% ($P = 0.7$), but increased root biomass of *P. lanceolata* by 18–38% ($P < 0.02$) across the CO_2 levels (Fig. 7b).

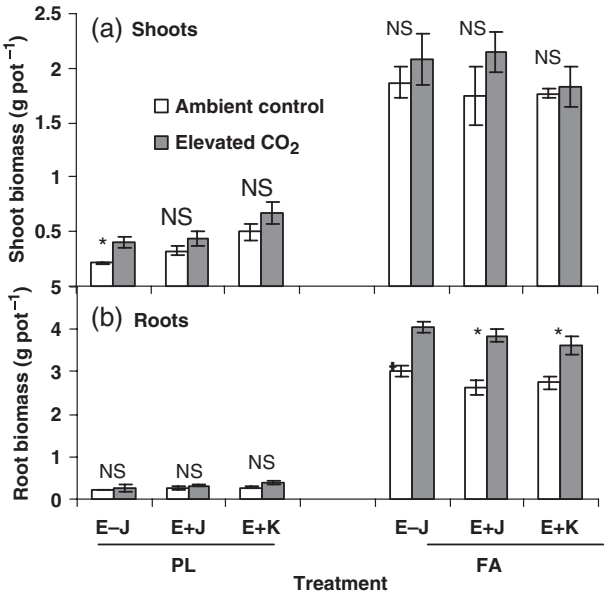

Fig. 7 Effects of fescue variety/endophytic status on shoot (a) and root (b) biomass of *Plantago lanceolata* (PL) and *Festuca arundinacea* (FA) in the mixture in Experiment 2. E−J, endophyte-free cv. Jesup; E+J, endophyte-infected cv. Jesup; E+K, endophyte-infected cv. Kentucky-31. Bars depict treatment means and standard errors (SE). *$P \leq 0.05$; NS, not significant between ambient and elevated CO_2.

Shoot biomass N and ^{15}N. Elevated CO_2 significantly reduced shoot N concentrations of both *P. lanceolata* and *F. arundinacea* (Table 1), but only led to a significant decrease in total shoot biomass N of

Table 1 Effects of elevated carbon dioxide (CO_2) on shoot ^{15}N and shoot N concentrations of *Plantago lanceolata* and *Festuca arundinacea* grown in mixture in Experiment 2

	^{15}N concentration ($\mu g\ ^{15}N\ kg^{-1}$)		N concentration ($mg\ N\ kg^{-1}$)	
Variety	ACO_2	ECO_2	ACO_2	ECO_2
P. lanceolata				
E−J	53.9 ± 3.31	71.0 ± 11.6	10.9 ± 0.36	10.8 ± 1.70
E+J	49.9 ± 1.25	43.5 ± 4.35	10.2 ± 0.56	8.26 ± 0.36*
E+K	59.5 ± 5.33	49.0 ± 7.64	11.7 ± 0.74	8.98 ± 1.35*
F. arundinacea				
E−J	44.4 ± 1.96	38.0 ± 2.92	10.4 ± 0.36	8.45 ± 0.44*
E+J	48.5 ± 0.51	38.8 ± 3.03	10.9 ± 0.31	8.30 ± 0.27*
E+K	56.6 ± 8.44	47.2 ± 6.43	11.4 ± 0.48	8.53 ± 0.33*

*Significantly different from the corresponding ambient control according to LSD test ($P \leq 0.05$).
ACO_2, ambient CO_2; ECO_2, elevated CO_2; N, nitrogen.

Table 2 Effects of elevated carbon dioxide (CO_2) on ^{15}N and N accumulation in the shoot biomass of *Plantago lanceolata* and *Festuca arundinacea* in mixture in Experiment 2

	Biomass ^{15}N ($\mu g\ N\ pot^{-1}$)			Biomass N ($mg\ N\ pot^{-1}$)		
Variety	ACO_2	ECO_2	Means†	ACO_2	ECO_2	Means
P. lanceolata						
E−J	11.5 ± 0.35	28.5 ± 5.33*	20.0b	2.35 ± 0.12	4.25 ± 0.66*	3.30b
E+J	15.9 ± 1.94	19.3 ± 3.97	17.6b	3.28 ± 0.49	3.60 ± 0.56	3.44b
E+K	29.1 ± 4.82	33.5 ± 7.80	31.3a	5.81 ± 1.01	6.22 ± 1.47	6.01a
F. arundinacea						
E−J	82.2 ± 3.53	77.2 ± 2.96	79.7a	19.3 ± 1.29	17.3 ± 1.28	18.3a
E+J	84.9 ± 12.9	82.4 ± 6.30	83.6a	19.2 ± 3.12	17.8 ± 1.36	18.5a
E+K	100.4 ± 16.5	86.0 ± 13.9	93.2a	20.1 ± 0.95	15.6 ± 1.78*	17.9a

†Means are averages of shoot biomass *N* in each fescue variety treatment under both ambient and elevated CO_2; Values followed by different letters within a plant are significantly different among the fescue varieties ($P \leq 0.05$).
*Significantly different from the corresponding ambient control within a measurement (LSD, $P \leq 0.05$).
ACO_2, Ambient CO_2; ECO_2, Elevated CO_2; N, nitrogen.

F. arundinacea in the PL∼E+K mixture (Table 2). CO_2 enrichment significantly increased total biomass N and biomass ^{15}N in *P. lanceolata* shoots in the absence of endophytes (i.e. E−J), but not in the presence of endophytes (i.e. E+J and E+K) (Table 2). The presence of endophytes in fescue plants did not influence shoot N concentrations of either plant species, but increased total shoot N of *P. lanceolata* with statistical significance in the E+K treatment (Table 2). No significant effects of endophytes were observed on the ^{15}N concentrations or total shoot ^{15}N of either plant species (by the E−J vs. E+J comparison) (Table 2).

Mycorrhizal colonization of plant roots and the presence of endophyte in tillers and seeds. Similar to what observed in the Experiment 1, mycorrhizal colonization of fescue roots was again very low (<3%) and was not affected by CO_2 levels and fescue varieties (data not shown). Likewise, fescue varieties (i.e. endophytic status) had no effects on the mycorrhizal root colonization and total colonization length of *P. lanceolata* under either CO_2 concentrations. However, elevated CO_2 enhanced the AM root colonization of *P. lanceolata* only when they were grown together with endophyte-free fescue plants (Fig. 6a and b). In addition, endophytic fungi were detected in all fescue plants in both E+J and E+K cultivars, but not in the E−J plants.

Effects of N availability on plant biomass, total plant N and plant ^{15}N content. High N input (35 $mg\ N\ kg^{-1}$ soil) significantly ($P < 0.05$) increased the biomass, N concentrations and total N contents, and ^{15}N contents of both *P. lanceolata* and *F. arundinacea* (data not shown).

However, no significant interactions were observed between N availability and endophytes.

Discussion

Results from our experiments showed that elevated CO_2 stimulated the growth of both plantago and tall fescue plants (Figs 1 and 7). However, the magnitude of the stimulation was significantly higher in plantago than in tall fescue, altering the relative composition of species biomass in mixture (Fig. 2). These results are consistent with a general pattern observed in other studies in which CO_2 enrichment favors forbs over grasses (Potvin & Vasseur, 1997; Leadley *et al*., 1999; Owensby *et al*., 1999; Teyssonneyre *et al*., 2002; Polley *et al*., 2003). The mechanisms that lead to higher responses of forb species than grasses to elevated CO_2 are not exactly known. However, results from our experiment indicate that mycorrhizae may significantly contribute to this differential response through altering nutrient acquisition of the coexisting plants. Compared with grasses, forb roots have a much smaller surface area per unit of root mass, potentially limiting their access to nutrients. CO_2-enhancement of mycorrhizae may, therefore, benefit forbs more than grasses in increasing root surface and nutrient interception, particularly of less mobile nutrients like P (Rouhier & Read, 1998; Syvertsen & Graham, 1999). Increased N and ^{15}N acquisition of plantago (Figs 3 and 4; Table 2) and reduced N in the Test compartments (Fig. 5) in our experiment provide direct evidence illustrating that elevated CO_2 promoted mycorrhizally mediated N uptake by *P. lanceolata* but not by fescue plants. This mycorrhizal modification of N allocation among coexisting plants can be important in understanding plant N acquisition and plant interaction under future CO_2 scenarios (Johnson *et al*., 2003; Hu *et al*., 2005).

One may argue that root exclusion through the nylon mesh in our microcosms is artificial, which does not represent the true situation in field soil where roots of mycorrhizal and nonmycorrhizal hosts enmesh each other. However, mycorrhizal hyphae may allow their hosts to access N and other nutrients in some microsites where plant roots are unable to reach (Schimel & Bennett, 2004). CO_2-enhancement of mycorrhizae may be a major mechanism through which plants increase nutrient interception and transport under elevated CO_2 (Hu *et al*., 2001, 2005). In addition, labile C inputs associated with mycorrhizal growth and turnover stimulate localized microbial activity and N mineralization (Hodge *et al*., 2001). Because of the vicinity of mycorrhizal hyphae to those hotspots, their hosts have the advantage to access this newly released N source (presumably other nutrients as well) over mycorrhizal-poor fescue plants.

It is very interesting to note that in our experiments, mycorrhizal root infection of both E+ and E− tall fescue varieties was extremely low and was not altered by elevated CO_2. Low mycorrhizal colonization of tall fescue roots has been attributed to the toxins produced by the endophyte symbionts (Chu-Chou *et al*., 1992; Guo *et al*., 1992; Müller, 2003). However, this assumption has not been vigorously tested. A recent study actually documented a positive interaction between *Neotyphodium* endophytes and arbuscular mycorrhizae in grass *Bromus setifolius* (Novas *et al*., 2005). Although toxin production was not ascertained in our experiment, low mycorrhizal infection in E− plants suggests that endophytic toxins might not directly contribute to mycorrhizal inhibition, at least in the varieties examined in our experiments.

Because both endophytes and mycorrhizae depend on the C sources from the hosts, it has also been suggested that competition for C sources between those two fungal groups exist (Vicari *et al*., 2002) and lower C availability in roots of endophytic plants might inhibit mycorrhizal infection and growth. Similarly, Vicari *et al*. (2002) showed that the beneficial effect of the foliar endophyte on perennial ryegrass, in terms of insect resistance, was reduced by mycorrhizae. When environmental stresses do not exist, endophytes might even behave as a C sink that reduces the competitiveness of host plants (Faeth & Fagan, 2002). If this hypothesis holds true, however, mycorrhizae would have taken advantage of enhanced C availability under elevated CO_2 or in the absence of endophyte in the host plants in our experiments. As the biomass remained statistically same for E+ and E− plants in Experiment 2, this 'C sink' hypothesis could not explain why the biomass of *P. lanceolata* was higher in the presence of E+ than E− plants. The alternative explanation is that E+ plants may increase resource availability for coexisting plants. Van Hecke *et al*. (2005) have recently shown significantly greater amount of soluble organic C and in turn higher microbial activity in the rhizosphere of E+ than E− tall fescue plants, enhancing nutrient mineralization and release from organic materials.

Responses of mycorrhizal and endophytic fungi to global change components may be important in understanding species interactions in many grasslands where mycorrhizal forbs and endophytic grasses coexist under future climatic conditions. On one hand, many grasses and forbs in grasslands are highly mycorrhizal (Smith *et al*., 1999; Johnson *et al*., 2003) and may confer additional competitive advantages over mycorrhizae-poor plants such as tall fescue under future CO_2 conditions, particularly in low nutrient soils. On the other hand, it

remains unclear how global change factors affect the activities of endophytic fungi. Our results suggest that endophytes may be less sensitive than mycorrhizae to elevated CO_2. Newman *et al*. (2003) observed that the presence of endophytes in tall fescue buffered their host response to elevated CO_2 by reducing the difference in the content of different crude proteins between ambient and elevated CO_2. Similarly, Hunt *et al*. (2005) have recently found that in the endophyte (*N. lolii)*-free ryegrass, soluble protein was significantly lower under elevated than ambient CO_2 and this difference was absent in endophyte-infected plants. In our experiments, plant biomass and N acquisition in *P. lanceolata* was less responsive to elevated CO_2 in the presence of endophytic tall fescue plants, although tall fescue plants were not affected (Fig. 7). In addition, it is well documented that endophytic fungi confer competitive advantage for host plants through enhancing their resistance to environmental stresses (Clay & Holah, 1999). Mineral N inputs have been shown to enhance toxin production by endophytic fungi in tall fescue (Belesky *et al*., 1988; Arechavaleta *et al*., 1992), but how the resulting changes influence mycorrhizal colonization and functioning remains unexplored. Together, these results indicate that the interactive effect of multiple global change components on mycorrhizae and foliar endophytes, and the resulting impact on species interactions warrant further investigation.

In summary, results obtained from our microcosm experiments showed that elevated CO_2 favored *P. lanceolata* over *F. arundinacea*, although the growth of both species was stimulated. Elevated CO_2 enhanced mycorrhizae in *P. lanceolata,* but did not have any significant effects on mycorrhizal colonization of *F. arundinacea* roots regardless of the status of endophytic infection. Neither the presence of endophytes nor mycorrhizal inoculation enhanced N acquisition and biomass accumulation in *F. arundinacea*. CO_2-enhancement of N acquisition in *P. lanceolata* through mycorrhizae likely predominates the differential effect of elevated CO_2 on two species. However, the role of endophytes in mediating the interactions between endophytic plants and their coexisting species under future CO_2 conditions remains unknown. Future studies are warranted to examine whether findings from this microcosm system hold true in field conditions and for other plant species and endophyte complexes under multiple environmental stresses.

Acknowledgements

We thank Howard Sanford for ^{15}N measurements and Lisa Lentz for total C and N determinations at the Soil Analytical Lab, NCSU. Thanks were also extended to Nick Hill and Jim Green for their constructive suggestions for experimental design, Jeff Barton for his technical help in maintaining the CSTR facility and to Betsy Randall-Schadel for her help in assessing the endophyte status in seeds and tillers of fescue plants. This study was supported by the Grants from the US Department of Agriculture (NRI-2000-00531) and the National Science Foundation (DEB-00-01686) to NC State University. Xin Chen was partially supported by a scholarship from the Pao Foundation Scholarship and a Grant from the National Science Foundation of China (NSFC, No. 30228005).

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