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Matthias C. Rillig · Michael F. Allen
John N. Klironomos · Nona R. Chiariello
Christopher B. Field

Plant species-specific changes in root-inhabiting fungi in a California annual grassland: responses to elevated CO_2 and nutrients

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Abstract Five co-occurring plant species from an annual mediterranean grassland were grown in monoculture for 4 months in pots inside open-top chambers at the Jasper Ridge Biological Preserve (San Mateo County, California). The plants were exposed to elevated atmospheric CO_2 and soil nutrient enrichment in a complete factorial experiment. The response of root-inhabiting non-mycorrhizal and arbuscular mycorrhizal fungi to the altered resource base depended strongly on the plant species. Elevated CO_2 and fertilization altered the ratio of non-mycorrhizal to mycorrhizal fungal colonization for some plant species, but not for others. Percent root infection by non-mycorrhizal fungi increased by over 500% for *Linanthus parviflorus* in elevated CO_2, but decreased by over 80% for *Bromus hordeaceus*. By contrast, the mean percent infection by mycorrhizal fungi increased in response to elevated CO_2 for all species, but significantly only for *Avena barbata* and *B. hordeaceus*. Percent infection by mycorrhizal fungi increased, decreased, or remained unchanged for different plant hosts in response to fertilization. There was evidence of a strong interaction between the two treatments for some plant species and non-mycorrhizal and mycorrhizal fungi. This study demonstrated plant species- and soil fertility-dependent shifts in belowground plant resource allocation to different morphogroups of fungal symbionts. This may have consequences for plant community responses to elevated CO_2 in this California grassland ecosystem.

Key words Elevated CO_2 · Arbuscular mycorrhiza · Saprophytic fungi · Soil nutrients · Annual grassland

M.C. Rillig (✉) · M.F. Allen
Department of Biology, Soil Ecology & Restoration Group, San Diego State University, San Diego, CA 92182, USA
Fax: (619)594-5676; e-mail: mrillig@sunstroke.sdsu.edu

J.N. Klironomos
Department of Botany, University of Guelph, Guelph, ON, Canada N1G 2W1

N.R. Chiariello
Department of Biological Sciences, Stanford University, Stanford, CA 94305, USA

C.B. Field
Department of Plant Biology, Carnegie Institution of Washington, Stanford, CA 94305, USA

Introduction

Understanding the response of plants and plant communities to the unprecedented rate of increase in the concentration of atmospheric carbon dioxide (Keeling et al. 1995) is a central theme of global change ecology (Houghton et al. 1990). Particularly important is the question whether a change in this basic resource will result in changes in plant community composition for a given ecosystem. Although data on differential effects of CO_2 on co-occuring plant species are becoming increasingly available (Körner et al. 1996), many studies on the effects of elevated CO_2 are limited to "model" organisms for certain ecosystems. This may lead to false generalizations when the amount of variability among the responses of different plant species is unknown (Körner et al. 1996).

Research has overwhelmingly concentrated on above ground responses to CO_2. However, there is evidence that rhizosphere organisms can provide crucial feedbacks to plants grown in elevated CO_2 (Diaz et al. 1993; Zak et al. 1993; O'Neill 1994). Root-colonizing fungi, through their direct plant tissue access, may be particularly susceptible to CO_2-induced changes in plant physiology. Due to their many different ecophysiological functions (Kendrick 1992), ranging from mutualism to parasitism from the plant's perspective, they may also provide a range of strong feedbacks to plant growth.

In global change biology, fungal research has focused on mycorrhizal fungi (O'Neill 1994; Sanders 1996; Klironomos et al. 1996). Arbuscular-mycorrhizal (AM) fungi are an important component of terrestrial nutrient cycles, because they are a sink for host photosynthate,

they are a component of the soil detrital food web, they translocate nutrients to the host, and they can mediate competition among plant hosts (Allen and Allen 1990; Allen 1991). Compared to the knowledge about plant responses to elevated carbon dioxide, the data base of AM fungal responses is very sparce (O'Neill 1994). So far it is difficult to predict symbiotic responses to elevated CO_2 because AM studies historically have focused on fungal benefits to the host (Whitbeck 1994; Allen 1996). Several studies have shown no effect of CO_2 on percent mycorrhizal colonization, although AM-colonized root length sometimes increased (O'Neill et al. 1991; Rogers et al. 1994; Runion et al. 1994; Whitbeck 1993, 1994; Monz et al. 1994). Monz et al. (1994) working with *Bouteloua gracilis*, Klironomos et al. (1996) with *Artemisia tridentata*, Jongen et al. (1996) with *Trifolium repens*, and Sanders (1996) with *Prunella vulgaris* observed increases in percent infection by AM fungal structures in response to elevated CO_2. Sanders (1996) documented a decrease in percent infection under elevated CO_2 in *P. grandiflora*. There is little evidence for an effect of CO_2 on fungi other than mycorrhizal fungi (Runion et al. 1994; Klironomos et al. 1996), and a lack of data on how responses can vary among plant species from the same community.

A main goal of the present research was to study the response of root-colonizing fungi to a crossed resource gradient of carbon dioxide and soil nutrient enrichment. Specifically, we tested the following hypotheses:

1. The percentage of roots infected with AM fungi will increase in elevated CO_2. Increases will be a function of plant species, with the biggest increases in plant species that have low ambient infection levels, and smaller increases in species with already high infection in ambient CO_2.

2. The percentage of roots infected with non-mycorrhizal (NM) fungi (melanized and non-melanized) will increase in elevated CO_2.

3. Fertilization with N, P, K will modify CO_2 responses for AM and NM fungi. In elevated CO_2, infection by AM fungi will be reduced by fertilization. The converse will be true for NM fungi.

Materials and methods

General

This research was part of the Jasper Ridge CO_2 project, located at the Jasper Ridge Biological Preserve near Stanford, California, United States (37°24′N, 122°13′W, 100 m elevation). The experiment was carried out at the outdoor MECCA (*M*icro*E*cosystems for *C*limate *C*hange *A*nalysis) facility using six open-top chambers (1.3 m^2) that received primarily natural rainfall inputs and either ambient or ambient plus 350 $\mu l\ l^{-1}$ CO_2. For further details on the experimental design and the chemical characteristics of the sandstone soil used in this study see Field et al. (1996). We grew monocultures of five plant species that co-occur in the mediterranean annual grassland (sandstone soil) in 7.6 × 7.6 × 50 cm pots. The plant species (Hickman 1993) consisted of three annual grasses (*Bromus hordeaceus* L., *Vulpia microstachys* (Nutt.) Benth. var. *pauciflora* (Beal) Lonard & Gould, *Avena barbata* Link) and two herbs (*Linanthus parviflorus* (Benth.) Greene, and *Calycadenia multiglandulosa* DC.). Per plant species, there were four treatment combinations, in which fertilization was crossed factorially with CO_2 concentration. Additional nutrients (20 g m^{-2} nitrogen, phosphorus and potassium) were supplied to half of the pots in each CO_2 treatment as 120-day time-release Osmocote fertilizer (Grace-Sierra Horticulture Product Company). Osmocote was added just before seeds were planted, and was placed at a depth of 1 cm below the soil surface to prevent surface growth of algae. To compensate for a late start of the growing season (due to delayed seasonal precipitation), all pots were hand watered several times in December 1995. After germination they received natural rainfall inputs.

Fungal root infection measurement

After 4 months, pots were destructively harvested, and roots were separated from the soil by wet-sieving. A random subsample of the roots in each pot was stored in 70% ethanol for subsequent analysis. Subsampled roots were cleared in 10% KOH (90°C) for 1 h, acidified in 1% HCl for 15 min, stained in 0.05% Trypan Blue in lactoglycerol (90°C) for 30 min, and then stored in lactoglycerol (Brundrett 1994). Fungal infection (at least ten 1-cm-long root fragments per sample) was measured with the magnified intersections method (McGonigle et al. 1990) at 200× magnification using the criteria described in Klironomos et al. (1996) and Miller et al. (1995). While there is still some debate on the separation of non-mycorrhizal and arbuscular mycorrhizal extraradical hyphae (Miller et al. 1995), intraradical hyphae of these groups of fungi can be distinguished morphologically, according to carefully described criteria (Gallaud 1905; Allen 1996; Klironomos et al. 1996; Widden 1996; Smith and Read 1997). AM fungal hyphae were distinguished from NM hyphae primarily by following a connection to AM structures like arbuscules (the defining criterion), coils and vesicles. Other, secondary factors we took into account were cross-wall septation (absent or irregular in AM hyphae), knobbiness of the hyphal wall (AM fungi), and hyphal branching (typically not at a right angle for AM hyphae). Non-AM hyphae were often connected to structures not found in the Glomales (AM fungi), e.g., conidia, which facilitated separation. Non-mycorrhizal hyphae were further divided into melanized and non-melanized hyphae, because hyphal melanization has been linked to increased drought resistance, structural stability, UV resistance, and anti-microbial properties (Cooke and Whipps 1993). Neither melanized nor non-melanized hyphae represent taxonomically defined groups of fungi, and even though the ecological characteristics of these broad groups have not been defined in the context of plant growth, future research may shed light on possible functional attributes. Both melanized and non-melanized hyphae can be indicative of saprotrophic and parasitic/pathogenic fungi. Total root lengths were measured according to Tennant (1975), and colonized root length was obtained by multiplying percent infection data with the root length (Allen et al. 1989). The proportion of root length colonized by different AM fungal structures (vesicles, arbuscules, coils, AM hyphae) was also quantified.

Data analysis

Percent root infection data was transformed using the arcsine of the square root to satisfy assumptions of normality (Shapiro-Wilks *W*-test) and homogeneity of variances (Bartlett-Fox *F*-test). Transformed data were then subjected to a 5 × 2 × 2 factorial multivariate ANOVA (MANOVA) for fungal hyphal (% infection) response variables (SAS Institute 1994). For each response variable, if the respective MANOVA model effect was significant, separate univariate ANOVAs were then calculated to test for treatment and plant host effects. Means were separated by Tukey-Kramer multiple comparison tests suitable for unequal sample

sizes, or with Dunnett's method when only the comparison with the control treatment was of interest.

Results

Evidence for the presence of a plant species dependency of fungal percent colonization with respect to fertilization and CO_2 treatments was provided by the significant $CO_2 \times$ species and fertilizer $\times$ species interaction terms and by the significant species main effect of a $5 \times 2 \times 2$ factorial MANOVA (Table 1). The fertilization treatment only weakly interacted with the CO_2 concentration, and there was a significant three-way interaction.

Subsequent univariate ANOVAs for the effects that were significant in the multivariate test (Table 2) revealed which hyphal types were responsible for the significant response. The plant species dependency in the fertilization treatment and the CO_2 treatment were caused by different fungal groups. The $CO_2 \times$ species interaction was significant for all three hyphal types, whereas the $CO_2 \times$ fertilizer interaction was significant

Table 1 Results from a $5 \times 2 \times 2$ factorial multivariate ANOVA with the response variables (arcsine square root transformed) arbuscular mycorrhizal (AM) hyphae infection (%), non-melanized NM hyphal infection (%), and melanized non-mycorrhizal (NM) hyphal infection (%). Pillai's trace was used as the multivariate criterion (*df* numerator, denominator degrees of freedom)

Model effect	Pillai's trace	*F*	*df*	*P*
CO_2	0.32	6.01	4, 51	0.0005
NPK	0.66	25.56	4, 51	< 0.0001
Species	1.74	10.39	16, 216	< 0.0001
$CO_2 \times$ NPK	0.18	2.94	4, 51	0.029
NPK $\times$ species	1.11	5.20	16, 216	< 0.0001
$CO_2 \times$ species	1.02	4.64	16, 216	< 0.0001
$CO_2 \times$ species $\times$ NPK	0.55	2.18	16, 216	0.006

Table 2 *F*-values for univariate ANOVAs for the response variables AM hyphal infection (%), non-melanized NM hyphal infection (%), melanized NM hyphal infection (%), and total NM hyphal infection (%)

Model effect	Mycorrhizal hyphae	Non-mycorrhizal hyphae		
		Non-melanized	Melanized	Total
CO_2	10.96*	2.91n.s.	5.28*	0.004n.s.
NPK	71.61***	0.002n.s.	32.85***	14.01***
Species	17.78***	25.43***	14.56***	18.50***
$CO_2 \times$ NPK	8.40**	2.38n.s.	0.71n.s.	2.02n.s.
NPK $\times$ species	19.46***	5.66***	2.32n.s.	5.77**
$CO_2 \times$ species	5.22**	6.21***	5.10***	16.47***
$CO_2 \times$ species $\times$ NPK	4.30**	2.61n.s.	1.70n.s.	3.04*

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, n.s. non-significant ($P > 0.05$)

Table 3 Means and SEM for the four treatment combinations, five plant species, and three response variables (AM hyphal infection, melanized, and non-melanized non-mycorrhizal (NM) hyphal infection). Behind the treatment combination (in parentheses) are given the number of replicates. Different letters (*a*, *b*, *c*) denote differences at $P < 0.05$ (Tukey-Kramer) within a response variable and species

		Arbuscular mycorrhizal hyphae	Melanized NM hyphae	Non-melanized NM hyphae
		Bromus		
Control	(4)	75.51 ± 2.91 a	25.38 ± 2.18 a	13.72 ± 2.45 a
CO_2	(4)	83.53 ± 2.98 a	16.31 ± 2.18 b	10.81 ± 2.31 a
NPK	(4)	40.43 ± 3.42 b	9.99 ± 1.59 b	25.80 ± 4.45 a
$CO_2 \times$ NPK	(4)	55.25 ± 3.35 c	8.84 ± 2.25 b	22.90 ± 6.37 a
		Calycadenia		
Control	(3)	51.18 ± 3.15 a	14.06 ± 0.54 a	18.25 ± 3.37 a
CO_2	(4)	57.13 ± 4.01 ab	9.04 ± 2.04 a	13.28 ± 4.24 a
NPK	(4)	40.85 ± 1.41 ac	2.40 ± 1.24 b	7.13 ± 1.91 a
$CO_2 \times$ NPK	(4)	43.17 ± 4.38 a	0.54 ± 0.39 b	3.46 ± 1.68 b
		Vulpia		
Control	(3)	41.47 ± 2.64 ab	18.85 ± 2.65 a	2.45 ± 1.89 a
CO_2	(3)	47.22 ± 2.29 a	6.13 ± 2.21 b	0.56 ± 0.56 a
NPK	(3)	59.91 ± 2.96 c	14.41 ± 2.42 a	1.33 ± 1.33 a
$CO_2 \times$ NPK	(2)	52.95 ± 3.67 a	2.41 ± 2.41 b	0.90 ± 0.90 a
		Linanthus		
Control	(4)	56.93 ± 4.76 a	1.26 ± 0.56 a	2.66 ± 2.03 a
CO_2	(4)	71.39 ± 1.68 b	10.91 ± 2.48 b	11.86 ± 4.48 a
NPK	(4)	41.12 ± 2.74 c	2.60 ± 2.60 a	5.68 ± 1.64 a
$CO_2 \times$ NPK	(4)	53.62 ± 4.90 a	1.42 ± 1.42 a	16.91 ± 2.94 b
		Avena		
Control	(4)	64.20 ± 3.71 a	4.54 ± 1.54 a	22.59 ± 4.13 a
CO_2	(4)	75.15 ± 2.26 b	5.46 ± 2.73 a	24.23 ± 5.01 a
NPK	(4)	64.61 ± 1.48 a	3.19 ± 1.28 a	8.70 ± 3.13 b
$CO_2 \times$ NPK	(4)	45.26 ± 2.96 c	5.80 ± 3.35 a	36.08 ± 3.86 c

only for AM hyphae. The CO_2 and fertilizer main effects were significant only for AM and melanized hyphae. The species main effect was significant for all hyphal types.

The trend for all five species was for an increase in the mean of AM percent infection in plants exposed to elevated CO_2. However, this increase was only statistically significant for *Linanthus* and *Avena* (Table 3). Percent infection by melanized hyphae sharply decreased for *Bromus* and *Vulpia*, increased for *Linanthus*, and did not change significantly for *Calycadenia* and *Avena*. None of the species showed a significant change in non-melanized hyphal infection as a result of the CO_2 treatment.

Fertilization elicited all three possible responses for AM colonization (Table 3): a significant decrease for *Bromus*, *Calycadenia*, and *Linanthus*, a significant increase for *Vulpia*, and no change for *Avena*. Melanized hyphal infection decreased for *Bromus* and *Calycadenia*, with the other species showing no significant change in infection.

Melanized and non-melanized fungal infection values are not additive, because frequently the different fungal groups formed overlapping areas of infection. Therefore we measured also the total non-mycorrhizal fungal infection. Percent change in AM fungal and total non-mycorrhizal infection for the different treatment combinations and plant species are contrasted in Fig. 1, demonstrating individualistic host-specific effects. Elevated CO_2 caused large and significant decreases in total NM fungal colonization for two plant species (*Bromus* and *Vulpia*). NM fungi colonizing *Avena* and *Calycadenia* responded only weakly to the CO_2 exposure, and NM fungal infection in *Linanthus* increased drastically. These different NM fungal responses were contrasted by a rather uniform increase in AM fungi across the plant species. The NM fungal colonization in response to fertilization and the crossed treatment provided further corroboration for individualistic host effects. A particularly clear interaction between the CO_2 and fertilization treatment was evident for *Bromus*, where each factor in isolation caused strong decreases in NM infection, whereas the simultaneous application elicited only a comparatively small change. There was no clear relationship across the hosts between AM and NM fungal behaviour.

Fertilization had a stimulating effect on total plant biomass for several hosts (data not shown), and consequently also on root biomass and length. Fungal-colonized root length (Figs. 2–3) therefore partially reflected those changes and modified the percent infection results accordingly. Elevated CO_2 did not significantly influence infected root length for mycorrhizal fungi in any host species. NPK application and the crossed treatments resulted in an increase in AM-colonized root length. For the NM fungi, the responses were more complicated, as fertilization resulted in an increase (*Bromus* and *Vulpia*), or no change in colonization. For *Calycadenia* there was no significant change in NM infection for any treatment.

Changes in mycorrhizal structures in response to the treatment combinations were evident for the different

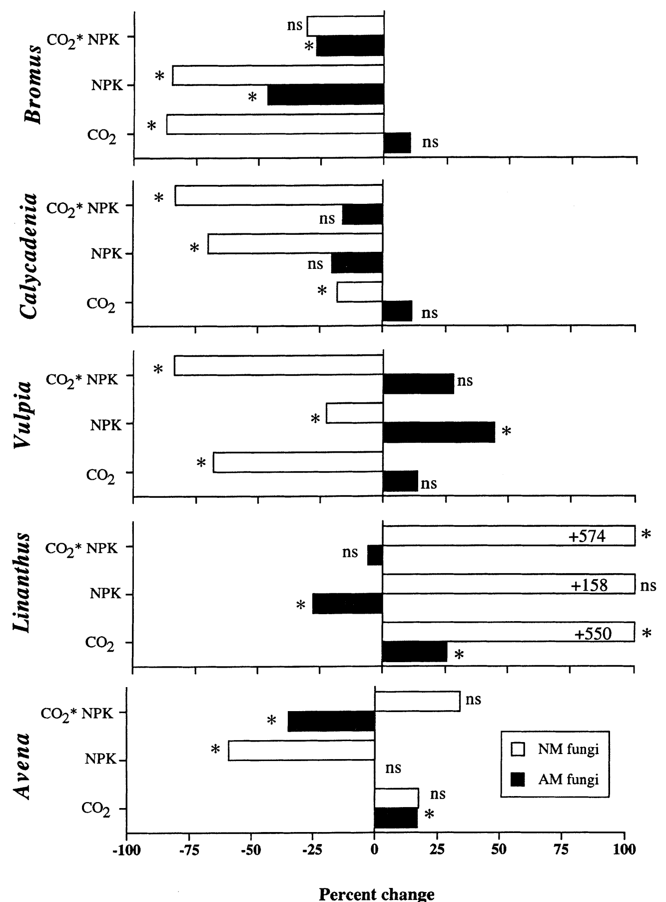

Fig. 1 Percent change (compared to control) of non-mycorrhizal (NM) fungal and arbuscular mycorrhizal (AM) fungal percent root infection of five plant species for the following treatments: elevated CO_2, fertilization (NPK), and elevated CO_2 and fertilization ($CO_2 \times$ NPK). *Asterisks* indicate significance at $P < 0.05$, *ns* indicates $P > 0.05$ (Dunnett's method)

plant species. Arbuscular infection did not mirror hyphal infection patterns for either CO_2 or fertilization (Fig. 4). For example, for *Bromus* and *Vulpia* there was an increase in arbuscules in elevated CO_2, although there was no significant change in % hyphal infection. For *Calycadenia* there was a decrease in arbuscular infection with no change in hyphal infection. There was no significant change in percent arbuscules in elevated CO_2 for *Linanthus* and *Vulpia*, but there was a significant increase in percent hyphal infection.

Discussion

This study provided strong evidence for a plant species dependent shift in root colonization by different fungal symbionts in response to elevated atmospheric CO_2, soil nutrient status and the CO_2/nutrient interaction. The five species differed markedly in percent mycorrhizal colonization under control conditions (Table 2). This provided an opportunity for a test of the hypothesis that plants

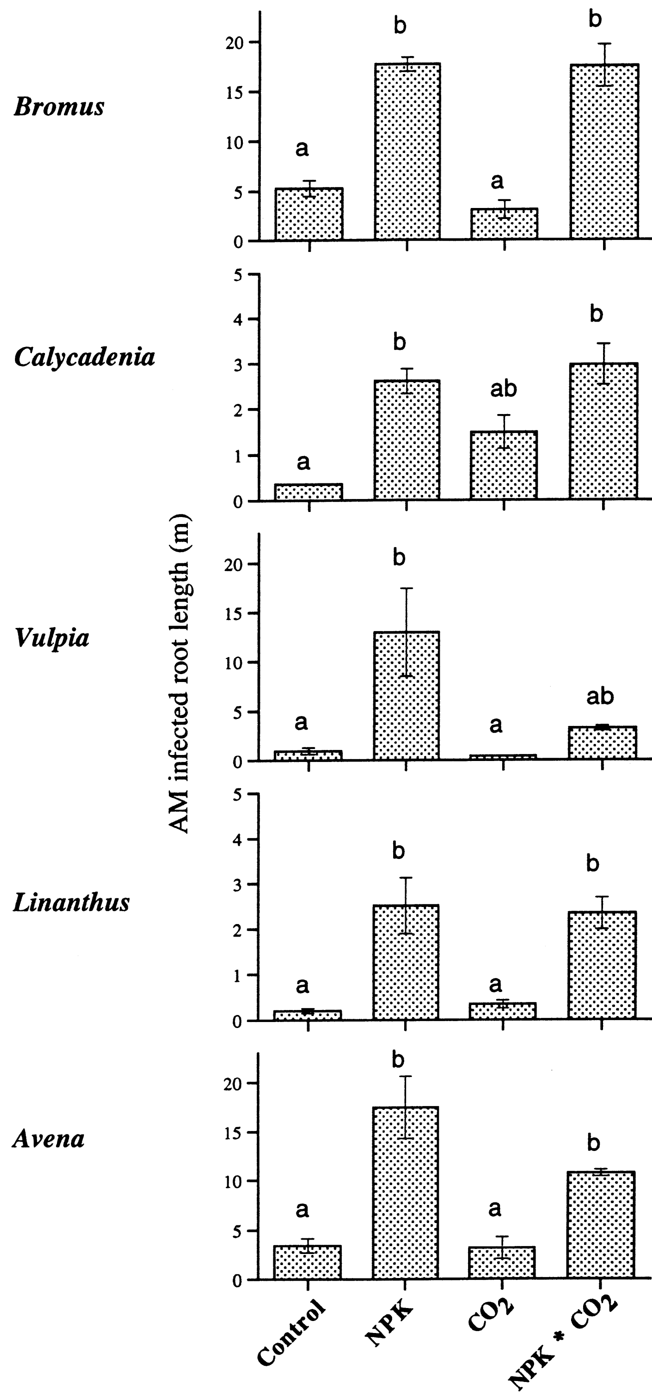

Fig. 2 Effects of elevated CO_2 and nutrient additions (NPK) on AM-infected root length (m) for five plant species. *Error bars* are SEMs. Different letters (*a, b*) represent differences at the significance level $P < 0.05$ (Tukey-Kramer)

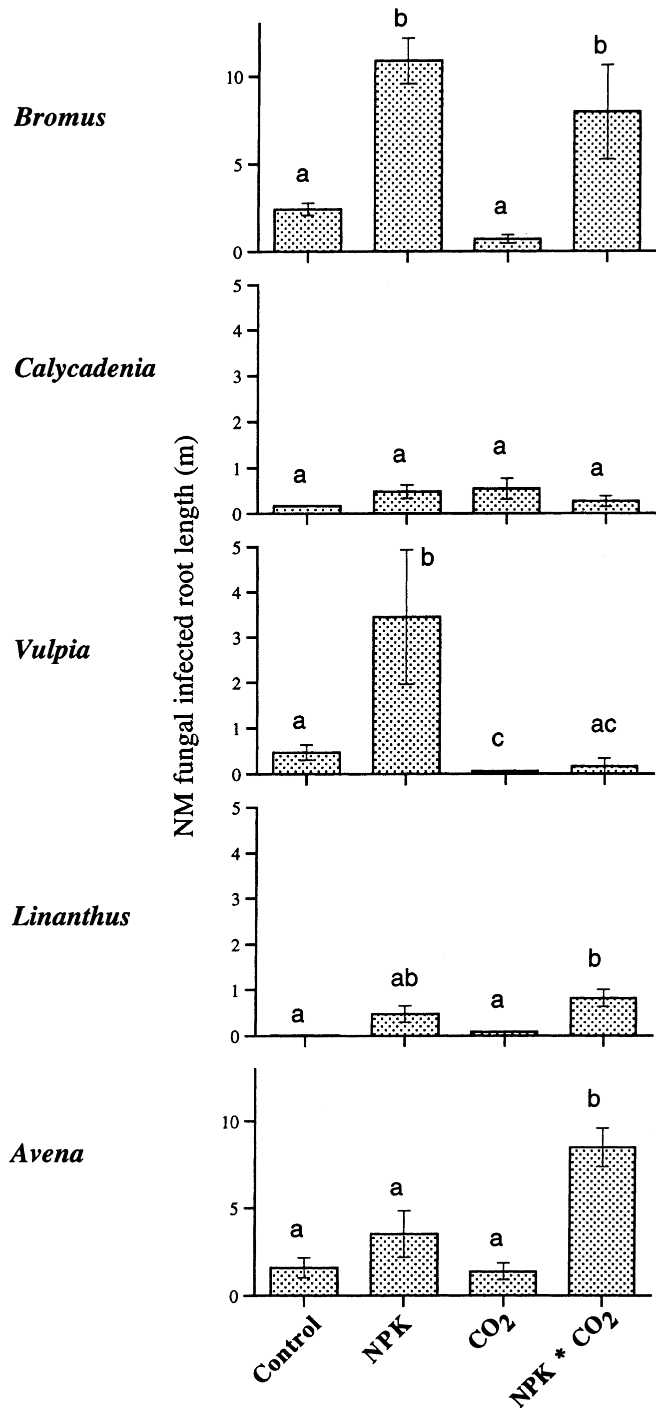

Fig. 3 Effects of elevated CO_2 and nutrient additions (NPK) on NM fungal infected root length (m) for five plant species. *Error bars* are SEMs. Different letters (*a, b, c*) represent differences at the significance level $P < 0.05$ (Tukey-Kramer)

with already very high levels of colonization are AM "saturated" (Allen et al. 1995), whereas hosts with lower levels of infection may show a strong increase in AM colonization in elevated CO_2. The data collected in this study do not completely support this hypothesis, because CO_2 should have elicited the greatest increase in percent AM infection in *Vulpia*, but in fact this was the case for *Linanthus*. However, *Bromus*, the species with the highest ambient percent AM colonization indeed showed the lowest proportional change when grown in elevated CO_2. Although ambient AM fungal colonization may play a role in determining the CO_2 response, it clearly is complicated by other factors. Interestingly, the only significant increase in AM colonization under elevated CO_2

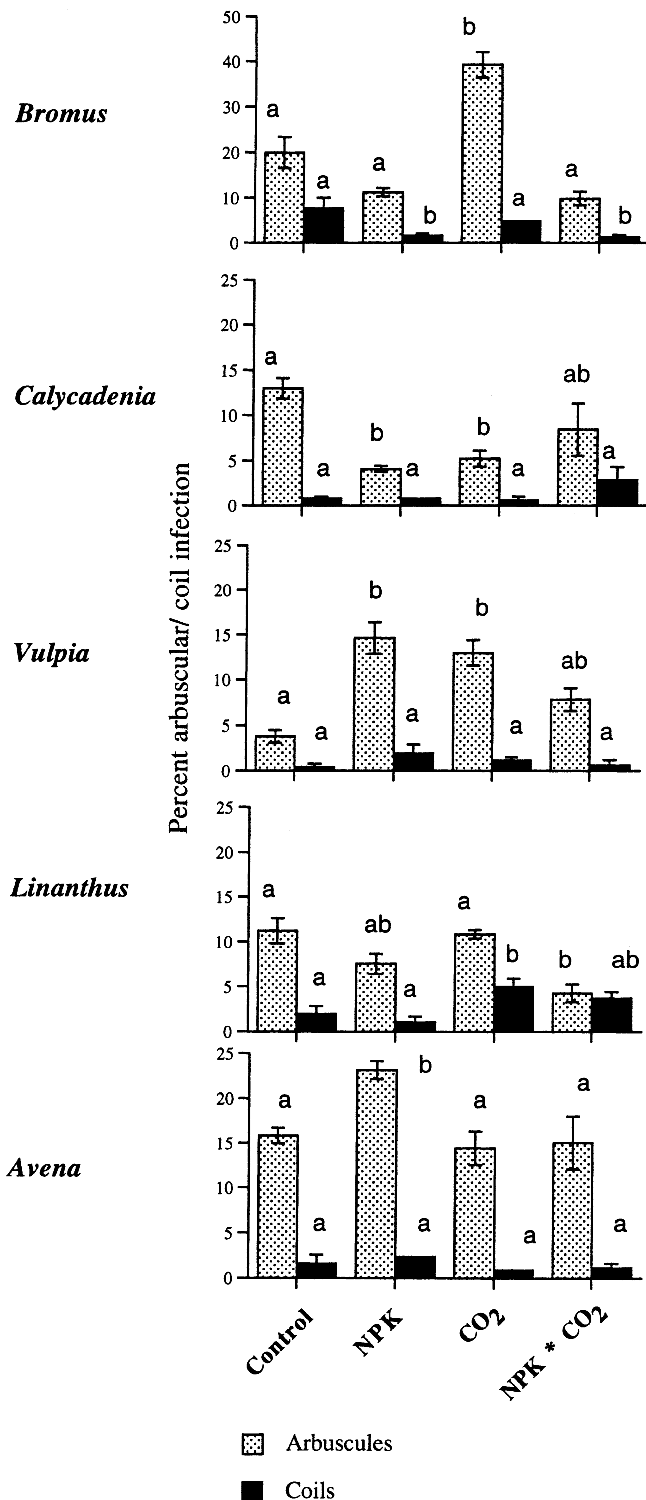

Fig. 4 Effects of elevated CO_2 and nutrient additions (NPK) on percent root infection by the AM fungal structures arbuscules and coils for five plant species. *Error bars* are SEMs. Different letters (*a, b*) represent differences at the significance level $P < 0.05$ (Tukey-Kramer)

occurred in the plant species that had the highest AM/NM fungal colonization ratio under control conditions: *Avena* and *Linanthus*. The other three species had lower ambient AM/NM ratios and there was no significant CO_2 response in AM colonization. The abundance of arbuscules and coils did not closely track the percent infection by AM hyphae, contrary to findings of a previous study (Klironomos et al. 1996). In the case of *Bromus* and *Vulpia* an increase in arbuscular infection provided evidence for an increase in AM fungal activity in elevated CO_2 in the absence of a significant hyphal infection response. However, despite an increase in hyphal infection for *Linanthus* and *Avena*, arbuscular infection remained unchanged. Infection by coils revealed no conspicuous pattern across the treatments, except that in *Bromus* coils were always reduced in the treatments with fertilization. This may reflect the fact that hyphal infection and the production of these nutrient/carbon exchange organs (Allen 1991) are under separate control.

Jackson and Reynolds (1996) found a down-regulation of the rates of nitrate uptake and no change for ammonium uptake for several plant species from a Jasper Ridge serpentine grassland grown under elevated CO_2. Since the N concentrations of these plants (including *Vulpia*, *Bromus*, and *Avena*) in root and shoot were relatively unaffected, uptake via the AM fungal symbionts may have increased. Although fungal nutrient translocation rates were not measured in our study, our results provide further support for this notion in that we found a tendency for an increase in hyphal infection for these plant species and increased arbuscular infection in *Bromus* and *Vulpia*. Arbuscules are the main sites of nutrient/carbon exchange between AM fungi and host (Smith and Read 1997).

We hypothesized that NM infection should increase in elevated CO_2 treatments: In this grassland photosynthetic CO_2 fixation increased in elevated CO_2 with only modest down-regulation (Fredeen et al. 1995; Jackson et al. 1995; Field et al. 1996), and an increase in fixed carbon typically leads to higher carbon allocation to roots (Runion et al. 1994), which should make roots richer sources of carbon (Rouhier et al. 1996). The observed response of NM fungi to CO_2 was therefore surprising. With the exception of *Linanthus*, no plant species in this study showed an increase of NM infection in response to CO_2, irrespective of the soil nutrient status. *Vulpia*, *Calycadenia* and *Bromus* showed very sharp decreases in NM infection in all CO_2 treatments. Runion et al. (1994) found a trend for infestation of rice with the fungal plant pathogen *Rhizoctonia* to be higher in elevated CO_2. Klironomos et al. (1996) reported no response to CO_2 in percent NM infection under low nutrient conditions using *Artemisia tridentata*, and a sharp increase in NM fungal infection under nutrient enrichment. The possibility that roots outgrew the fungi in our study can be discounted because root lengths did not increase in elevated CO_2 (data not shown). It is also unlikely that plant phenology was a confounding factor. Elevated CO_2 has been found to speed up the life cycle of plants (Garbutt and Bazzaz 1984), but infection by saprobes/pathogens increases, not decreases, with the progression of the life cycle of a plant (Parkinson and

Thomas 1969). Other possible explanations may include direct AM/NM fungal interactions (Newsham et al. 1995), or competition between mycorrhizal plants and NM fungi for nutrients (Diaz et al. 1993).

There are numerous studies on the responses of arbuscular mycorrhizal infection to nutrient additions. Most studies report a decrease in mycorrhizal infection following nutrient additions, for example of nitrogen (Chambers et al. 1980; Johnson et al. 1984). However, there are also reports of increases or no changes in AM fungal infection with fertilization (Heijne et al. 1992, 1994). To our knowledge, there is no study that reports all these responses for co-occurring plant species subjected to the same nutrient enrichment treatment. This result implies that percent AM root infection may be strongly controlled by the plant.

Studying CO_2 responses of a plant community from the same grassland, Whitbeck (1994) concluded that there was no significant effect of elevated CO_2 on AM infection. Plant species were not examined separately in that study, but pooled for a community estimate of colonization. This may have obscured significant effects for some of the species examined. Had we only measured mycorrhizal infection, our conclusion pertaining to a CO_2 effect may have been very similar. Our work therefore demonstrates that it is necessary to obtain data on several plant species when the ecosystem consequences of elevated CO_2 on fungal infection patterns are to be evaluated. Structure-function relationships are difficult to establish for root fungal symbionts, and therefore a concise evaluation of feedbacks to plant growth from the observed changes in abundance of fungal morpho-groups is not possible. For example, while higher levels of AM fungi in roots may be related to increased fungal nutrient transport (Smith and Read 1997), it is possible that the treatments caused shifts in the root-infecting AM fungal community, which could lead to different average nutrient translocation efficiencies (Jakobsen 1995). However, our results indicate that shifts in resource allocation to fungal symbionts, as a function of plant species, are a potentially important topic of plant community level research.

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