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Arbuscular mycorrhizae respond to plants exposed to elevated atmospheric CO_2 as a function of soil depth

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Abstract

The importance of arbuscular mycorrhizae (AM) in plant and ecosystem responses to global changes, e.g. elevated atmospheric CO_2, is widely acknowledged. Frequently, increases in AM root colonization occur in response to increased CO_2, but also the lack of significant changes has been reported. The goal of this study was to test whether arbuscular mycorrhizae (root colonization and composition of root colonization) respond to plants grown in elevated CO_2 as a function of soil depth. We grew *Bromus hordeaceus* L. and *Lotus wrangelianus* Fischer & C. Meyer monocultures in large pots with a synthetic serpentine soil profile for 4 yr in an experiment, in which CO_2 concentration was crossed factorially with NPK fertilization. When analyzing root infection separately for topsoil (0–15 cm) and subsoil (15–45 cm), we found large (e.g., about 5-fold) increases of AM fungal root colonization in the subsoil in response to CO_2, but no significant changes in the corresponding topsoil of *Bromus*. Only the coarse endophyte AM fungi, not the fine endophyte AM fungi, were responsible for the observed increase in the bottom soil layer, indicating a depth-dependent shift in the AM community colonizing the roots, even at this coarse morphological level. Other response variables also had significant soil layer * CO_2 interaction terms. The subsoil response would have been hidden in an unstratified assessment of the total root system, since most of the root length was concentrated in the top soil layer. The increased presence of mycorrhizae in roots deeper in the soil should be considered in sampling protocols, as it may be indicative of changed patterns of nutrient acquisition and carbon sequestration.

Introduction

Arbuscular mycorrhizae (AM), ubiquitous mutualistic symbioses between the roots of the vast majority of land plants (Allen, 1991) and fungi in the Glomeromycota, are an important factor to consider in attempts to understand the effects of elevated atmospheric CO_2 on plants and ecosystems (Fitter et al., 2000; Hodge, 1996; Rillig and Allen, 1999; Rillig et al., 2002; Treseder and Allen, 2000). Most likely due to the increased availability of carbon to the mycobionts, and the increased demand for services provided by the fungus (e.g. nutrient translocation), a commonly observed response to CO_2 enrichment is increased percent root colonization or mycorrhizal colonized root length (Rillig et al., 2002). However, there are also reports of no significant changes occurring in response to elevated CO_2 (reviewed in Rillig et al., 2002).

In soil ecology, lack of statistical power brought about by high variability and, sometimes, low sample sizes, can be responsible for failing to reject null hypotheses; this problem can sometimes be remedied by suitably stratified sampling (e.g., Klironomos et al., 1999). In this study we wished to examine whether mycorrhizal responses to elevated CO_2 could be more

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clearly revealed by analyzing root samples separately by depth.

In many studies of elevated CO_2 effects, assessment of mycorrhizae in pot experiments was limited to either a random subsample of the whole root system (e.g., Godbold et al., 1997; Jongen et al., 1996; Klironomos et al., 1998; Lovelock et al., 1996; Rouhier and Read, 1998; Sanders et al., 1998; Syvertsen and Graham, 1999) or a sampling of the top several centimeters of soil and roots (e.g., Dhillion et al., 1996; Klironomos et al., 1996; Monz et al., 1994). Staddon et al. (1998) measured root colonization responses of plants grown in ambient and elevated CO_2 at several depths. However, having applied AM fungal inoculum as a narrow band near the soil surface, the authors acknowledged that it was difficult to draw strong conclusions concerning a depth-dependent response. Morgan et al. (1994) also collected samples of roots from several depths, but mixed and pooled them prior to analysis, hence losing any depth-dependent information.

In field experiments, mycorrhizal infection was often only measured for the top few centimeters of soil for practical reasons (Klironomos et al., 1997; Rillig et al., 1999a, b, 2000), or it was unclear how roots for mycorrhizal assessment were sampled. When deeper soil cores were taken, mycorrhizae were examined on pooled root samples across the entire depth (Runion et al., 1994). It follows that we have only a very limited understanding of how arbuscular mycorrhizae respond as a function of soil depth to plants exposed to elevated atmospheric carbon dioxide (or other factors).

Conversely, root responses of plants grown in elevated atmospheric CO_2 have frequently been measured for several soil depths, probably in part because non-destructive methods for measuring root lengths (i.e., minirhizotron observations) exist, compared to the destructive assessment of mycorrhiza variables. In many cases, the elevated CO_2 treatment was found to stimulate root growth more strongly near the soil surface than deeper in the soil profile (e.g., Arnone et al., 2000; Fitter et al., 1997; VanVuuren et al., 1997; Prior et al., 1994). However, relatively even root growth stimulation throughout the rooting profile has also been documented (e.g. Rogers et al., 1992), and Day et al. (1996) documented an increase in fine roots near the surface and at greater depth (50–60 cm).

We were interested in testing whether AM fungi, like roots, can respond to plants exposed to elevated atmospheric CO_2 as a function of soil depth. Two alternative hypotheses presented themselves. AM fungal responses could follow root responses in being frequently more pronounced near the soil surface. This would be consistent with the idea that the part of the root system closest to the source of recently fixed carbon should be able to support a higher level of mycorrhizal colonization (Staddon et al., 1998). Alternatively, roots could be more 'mycorrhiza-saturated' near the surface, but greater potential could exist for increased host carbon allocation to AM fungi in the deeper soil layers.

To test these hypotheses, we used plants and soil from a serpentine grassland ecosystem, for which it is known that AM fungal inoculum is spatially structured, horizontally (Whitbeck, 1994), as well as vertically (Koide and Mooney, 1987). We examined these ideas for two plant species under two different nutrient conditions (the latter was included since previous experiments had shown that nutrient concentrations are an important potential modifier of AM fungal response to elevated CO_2 (e.g. Klironomos et al., 1996)).

Materials and methods

Experimental design, plant growth conditions and harvest

Bromus hordeaceus L. and *Lotus wrangelianus* Fischer & C. Meyer (Hickman, 1993), both annuals, were grown in monoculture in 0.9 m deep tubes (0.2 m diameter) inside 14 of the open-top chambers (1.3 m^2) at the outdoor Jasper Ridge MECCA (*M*icro*E*cosystems for *C*limate *C*hange *A*nalysis) facility near Stanford, Calif., USA (37° 24′ N, 122° 13′ W, 100 m elevation). The soil profile used was a synthetic serpentine soil profile consisting of topsoil (0–15 cm) and subsoil (15–90 cm) of crushed rock. This mimics the field situation (Field et al., 1996). The experiment was carried out in pots, since due to the rockiness of the soil it is virtually impossible to core deeper than 15 cm. For a detailed description of the MECCA set-up and a chemical characterization of the soils used in this study see Field et al. (1996).

This experiment had a complete factorial design with the factors CO_2 concentration and NPK fertilization. Elevated atmospheric CO_2 concentration was controlled at ambient plus 350 μL l^{-1} CO_2. Additional nutrients (nitrogen, phosphorus and potassium) were supplied to half of the pots in each CO_2 treatment as 120-day time-release Osmocote fertilizer (20 g m^{-2}; Grace-Sierra Horticulture Product Company).

Table 1. Lotus: *F* and *P* values from 2×2×2 factorial (Layer, NPK-fertilization, and atmospheric CO_2 concentration) analysis of variance or χ^2 and *P* values from Kruskall-Wallis tests. Significant *P* values are bolded, marginally significant *P* values ($P < 0.1$) italicized. No fine endophyte AM hyphae were observed in *Lotus*, hence effects could not be tested

Response variable	Layer		NPK		CO_2		Layer*NPK		Layer*CO_2		NPK*CO_2		Layer*NPK*CO_2	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
AMF total colonization (%)	148	<**0.0001**	55.8	<**0.0001**	0.43	0.51	7.33	**0.01**	4.94	**0.04**	4.17	*0.05*	0.42	0.52
Arbuscular colonization (%)	$\chi^2 = 21.3$ (d.f. =7); $P = 0.003$													
Coarse AMF colonization (%)[a]	148	<**0.0001**	55.8	<**0.0001**	0.43	0.51	7.33	**0.01**	4.94	**0.04**	4.17	*0.05*	0.42	0.52
Fine AMF colonization (%)	No fine hyphae observed in *Lotus*													
Root length (m)	2.56	0.12	18.6	**0.0003**	15.9	**0.0006**	3.79	*0.06*	0.04	0.84	4.65	**0.04**	1.99	0.17
AMF root length (m)	$\chi^2 = 24.9$ (d.f. =7); $P = 0.0008$													

[a] arc-sine square root transformed.

There were four replicate pots per plant species and treatment combination, except for the following for which there were only 3: *Bromus* layer 1, NPK− CO_2+, layer 1 NPK+ CO_2+, and layer 2 NPK− CO_2+; *Lotus*: layer 1 NPK− CO_2−, layer 2 NKP− CO_2+. There was an additional replicate (i.e. 5) for the following 3 treatment combinations: *Bromus* layer 1 NPK− CO_2−, *Lotus*: layer 1 NPK− CO_2+, layer 1 NPK+ CO_2+. Plants were grown for four years and pots were allowed to re-seed every year. The starting density for the monocultures was designed to resemble field densities (*Bromus*: 60 plants per pot; *Lotus* pots were re-seeded in the second year of the experiment to yield a density of 10 plants per pot). The experiment was run for several years partly to ensure recovery of the mycorrhizal hyphal network, which was likely disrupted during soil preparation.

At the time of harvest (April 1996), soil was ejected from the tubes and cut into three soil layers (0–15 cm, 15–45 cm, 45–90 cm). Since the 45–90 cm layer never contained a significant amount of roots, and no mycorrhizal fungi (data not shown), it was excluded from this study. From each of the layers, roots were obtained by washing and wet sieving. Roots of occasionally occurring weeds were carefully removed and excluded from analysis. A subsample of roots (obtained after thoroughly mixing all roots within a layer) consisting of 15–20 2-cm long pieces of fine roots was stored in 50% ethanol for fungal root infection measurements.

Root and mycorrhiza analyses

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 10 1-cm-long root fragments per sample) was measured with the magnified intersections method (McGonigle et al., 1990) at 200 × magnification using the criteria for differentiation of mycorrhizal and non-mycorrhizal hyphae described in Rillig et al. (1999b). Additionally, two types of AM hyphae were scored separately. Fine endophyte (FE) mycorrhizal hyphae (e.g. Gianinazzi-Pearson et al., 1981) were distinguished from coarse AM hyphae by means of hyphal diameter (1–2 μm for FE, contrasted with 3–10 μm for coarse hyphae), vesicles, staining with Trypan Blue (intensely blue for FE), and general growth pattern in roots, as described in Rillig et al. (1999b). We also separately measured percent root colonization with arbuscules.

Root lengths for each sample and soil layer were measured using a grid-line intersect method according to Tennant (1975) on a subsample of roots (approx. 0.15 g; three replicate counts per sample were performed and then averaged). Infected root length for each layer and sample was obtained by multiplying the percent infection value with root length.

Table 2. Bromus: *F* and *P* values from 2x2x2 factorial (Layer, NPK-fertilization, and atmospheric CO_2 concentration) analysis of variance or χ^2 and *P* values from Kruskall-Wallis tests. Significant *P* values are bolded, marginally significant *P* values ($P < 0.1$) italicized

Response variable	Layer		NPK		CO_2		Layer*NPK		Layer*CO_2		NPK*CO_2		Layer*NPK*CO_2	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
AMF total colonization (%)	42.1	**<0.0001**	10.86	**0.003**	10.51	**0.004**	2.63	0.12	1.88	0.18	2.39	0.13	7.19	**0.01**
Arbuscular colonization (%)	$\chi^2 = 14.1$ (d.f. =7); $P = 0.05$													
Coarse AMF colonization (%)	18.5	**0.0003**	13.54	**0.001**	4.76	**0.04**	0.13	0.72	4.21	*0.05*	3.49	*0.08*	4.12	*0.05*
Fine AMF colonization (%)[a]	19.8	**0.0002**	2.54	0.13	0.17	0.68	5.26	**0.03**	0.87	0.36	0.002	0.97	0.002	0.96
Root length (m)[b]	69.3	**<0.0001**	111	**<0.0001**	2.81	0.110.81	0.38	0.69	0.42	0.78	0.38	0.0003	0.96	
AMF root length (m)	$\chi^2 = 24.8$ (d.f. =7); $P = 0.0008$													

[a]arc-sine square root transformed.
[b]log transformed.

Data analysis

Data were analyzed using $2\times2\times2$ factorial analysis of variance with the fixed factors layer, CO_2 concentration, and fertilizer (NPK) addition. Data were analyzed separately for the two plant species, since *Lotus* pots had to be re-seeded in the second year, and since the two species were planted at different densities (as justified above). Assumptions of normality (Shapiro-Wilks W test) and homogeneity of variances (Levene's test) were checked for each test, and data were transformed (log or arc-since square root) if necessary. If data could not be transformed to meet assumptions of ANOVA, we employed a non-parametric test (Kruskall-Wallis). Statistical significance was accepted for $P \leq 0.05$, trends are described for $P < 0.1$.

Results

Responses in Lotus

In *Lotus*, no significant response in total AM root colonization to elevated CO_2 occurred in the top soil layer, irrespective of NPK fertilization (Figure 1, Table 1). However, the CO_2 * layer interaction term was significant for this response variable (Table 1), and there was an increase in root colonization in the bottom layer (Figure 1). Data for arbuscular colonization were analyzed with the non-parametric test, not permitting for a test of the interaction term; however the P value for the Kruskall-Wallis test was significant (Table 1), and no striking depth-dependence to a CO_2 response was apparent (Figure 1). NPK fertilization reduced both total AM root colonization and also arbuscular colonization, and for total AM root colonization the CO_2 * soil layer interaction was highly significant (Table 1). In *Lotus*, only coarse AM hyphae occurred, and hence no fine endophyte AM responses could be analyzed (Figure 2). Figure 3 shows root length and AM colonized root length responses. Root length was increased in elevated CO_2 and in the fertilized pots. There was no significant CO_2 * soil layer interaction, but the NPK * soil layer interaction was marginally significant. Total AM colonized root length followed a similar pattern, although we could not test for an interaction in the latter case (Table 1).

Responses in Bromus

There was a significant three-way interaction for total AM root colonization and a marginally significant three way interaction for just coarse AM root colonization (Table 2), indicating that the CO_2 response depended on both NPK and soil layer. In the top layer, elevated CO_2 only increased root colonization in the NPK-fertilized pots; however, in the bottom layer, there was a more than 5-fold increase in root colonization in the non-fertilized plants. Although we could not test for interaction terms for arbuscular colonization, there was an overall marginally significant effect (Table 2); we noted an increase in the bottom layer non-fertilized plants as well. Breaking down coloniza-

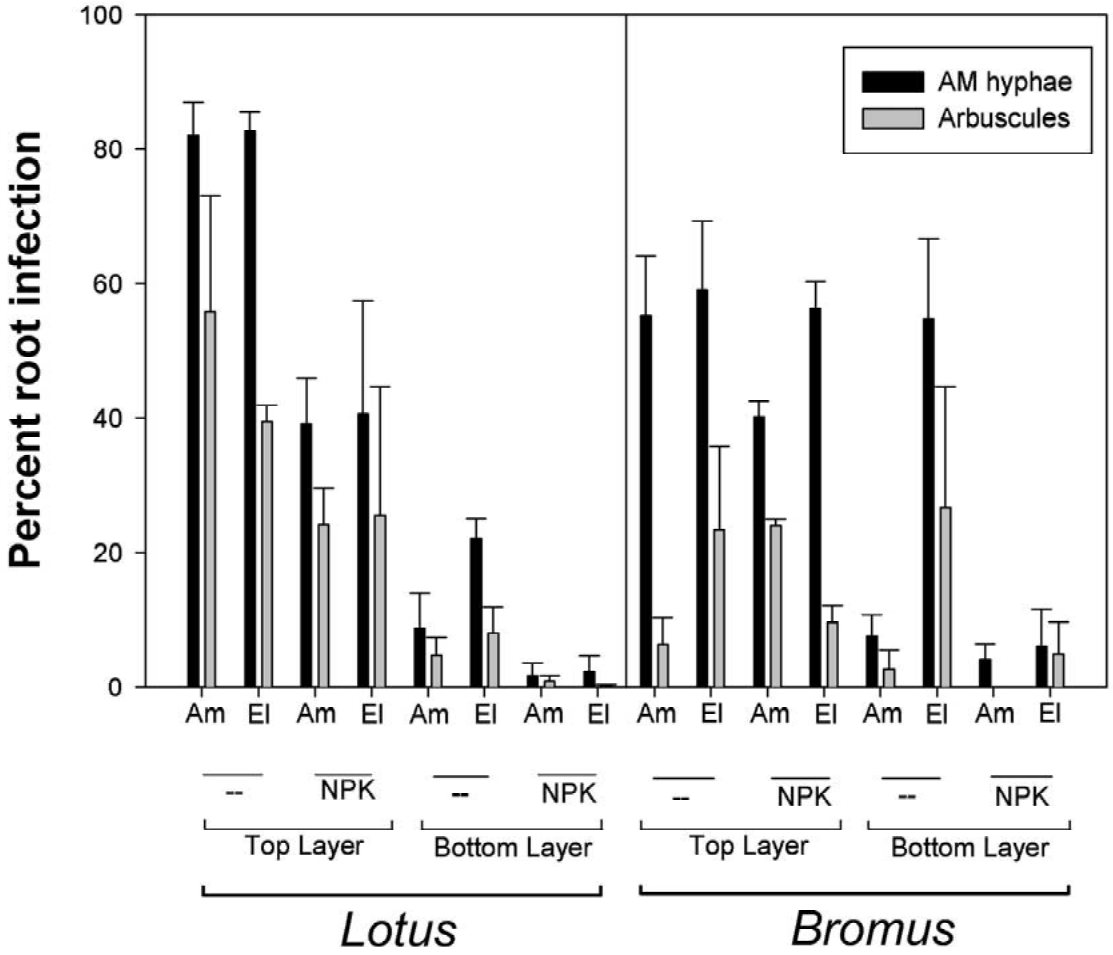

Figure 1. Effects of elevated (El) and ambient (Am) atmospheric CO_2 concentration and NPK fertilization on arbuscular mycorrhizal (hyphal) total percent root infection and arbuscular root infection at two soil depths (top layer: 0–15 cm, bottom layer 15–45 cm) in *L. wrangelianus* and *B. hordeaceus*. Error bars are one standard error of the mean (see 'Methods' for *n*).

tion responses into fine endophyte AM and coarse AM colonization (Figure 2), it was apparent that the former did not significantly respond to the CO_2 treatment, while the latter were responsible for observed changes at the total AM root colonization level (Table 2). For example, the notable increase in root colonization in the bottom soil layer of non-fertilized plants was entirely due to coarse AM hyphae. Overall, fine endophyte AM root colonization was lower in the bottom soil layer (Table 2), as was the case for coarse AM colonization. Root length in *Bromus* did not significantly respond to CO_2 level ($P = 0.11$) (Table 2). We could not test for interactions for AM colonized root length, but the overall effect was significant.

Discussion

Arbuscular mycorrhizal responses to plants exposed to elevated atmospheric CO_2 followed a soil-depth dependent pattern in this study, as evidenced by significant interactions of CO_2 with soil layer depth. The most striking observation of the study is perhaps the large increase in colonization of coarse AM hyphae in the lower soil layer in *Bromus*. For the non-fertilized pots, there was no significant CO_2 effect in the corresponding top soil layer. This did not support the hypothesis that the greatest responses occurs in the part of the root system closest to the source of the most recently fixed carbon. However, since there was a trend for higher root length in the top soil layer under elevated CO_2, there was in fact a higher total amount

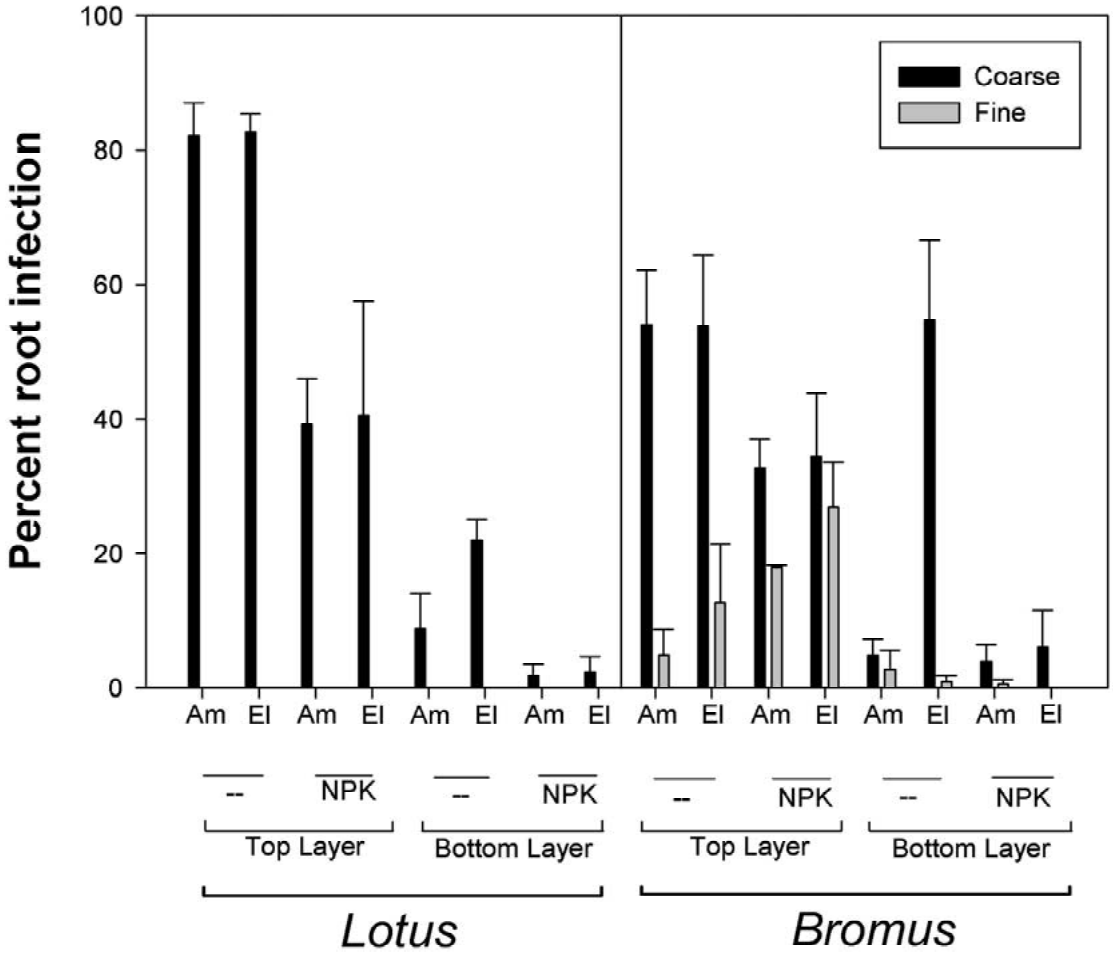

Figure 2. Effects of elevated (El) and ambient (Am) atmospheric CO_2 concentration and NPK fertilization on arbuscular mycorrhizal percent root infection by coarse and fine endophyte fungi at two soil depths (top layer: 0–15cm, bottom layer 15–45 cm) in *L. wrangelianus* and *B. hordeaceus*. Error bars are one standard error of the mean (see 'Methods' for *n*).

of mycorrhizae in this layer as well, which can be seen as a mycorrhizal response in itself (O'Neill, 1994).

Interestingly, root length itself did not respond to elevated CO_2 in the bottom layer of *Bromus* plants (Figure 3), while concurrently the large colonization increase occurred. This may suggest that increased mycorrhizal colonization (and colonization by arbuscules, the organs of nutrient-carbon exchange) could contribute to enhanced root exploration of the bottom soil layer, but physiological studies are necessary to provide direct evidence for this hypothesis. In our study root length was much lower in the bottom soil layer compared to the top soil layer (which is often used as a justification of sampling only the top several cm in the field). The relative importance of the observed increase in root colonization in the bottom soil layer (with the low root biomass) to the whole root systems and plant nutrient and/ or water budget is hence not clear.

Having distinguished coarse and fine endophyte AM root colonization, we were able to add to the growing evidence that elevated CO_2 can differentially stimulate AM fungi. As we previously showed in the field (in the grassland from which plants for the present study were obtained), fine endophyte AM root colonization was typically not increased under elevated CO_2 (Rillig et al., 1999b). This differential responsiveness to CO_2 was most convincingly demonstrated in the stimulation of coarse AM colonization in *Bromus*, bottom soil layer non-fertilized plants, with fine endophyte hyphae not affected. Klironomos et al. (1998) have also directly shown that different

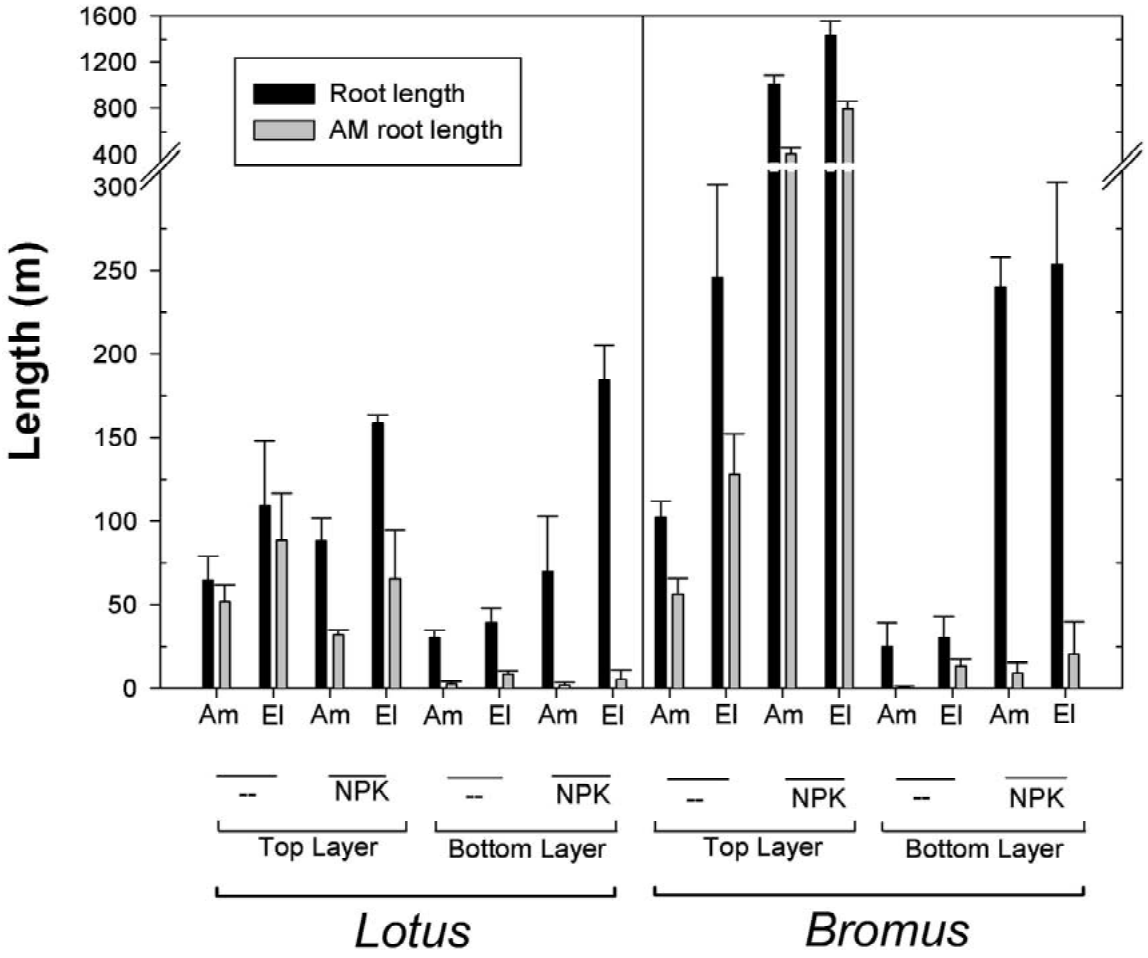

Figure 3. Effects of elevated (El) and ambient (Am) atmospheric CO_2 concentration and NPK fertilization on root length and arbuscular mycorrhizal root length for two soil depths (top layer: 0–15 cm, bottom layer 15–45 cm) in *L. wrangelianus* and *B. hordeaceus*. Error bars are one standard error of the mean (see 'Methods' for *n*). Note y-axis break.

AM fungal isolates can respond differently to CO_2 exposure.

As others and we have shown previously for other systems (reviewed in Rillig et al., 2002), soil fertility can significantly affect root colonization and alter the mycorrhizal response to CO_2. Several NPK * CO_2 interaction terms were significant for *Lotus*, and in *Bromus* the 3-way interaction term Layer * NPK * CO_2 was significant for AM total colonization and coarse AM root colonization (Tables 1 and 2). NPK fertilization had strong positive effects on root lengths in the bottom soil layer in our study, as a consequence of higher plant biomass (data not shown). However, owing to the generally low root colonization, this did not result in high mycorrhizal root length in the bottom soil layer.

Our results indicate that sampling only the top soil layer for mycorrhizae may underestimate responses of the symbiosis to elevated CO_2 exposure. If we had only sampled the top 15 cm of soil (in the non-fertilized plants) the conclusion of the study would have likely been that there was no mycorrhizal response to CO_2 for both plant species. This would argue for a stratified sampling rather than the much more common random root sample approach. This result also suggests that many non-significant mycorrhizal responses to CO_2 may have been partially a result of sampling protocol, if soil-depth dependent responses occur in other ecosystems as well.

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