Soil nutrient heterogeneity interacts with elevated CO_2 and nutrient availability to determine species and assemblage responses in a model grassland community

Fernando T. Maestre[1]*, Mark A. Bradford[2] and James F. Reynolds[1,3]

[1]Department of Biology, Duke University, Phytotron Building, Box 90340, Durham, NC 27708, USA; [2]Institute of Ecology, University of Georgia, Athens, GA 30602, USA; [3]Nicholas School of the Environment, Division of Environmental Science & Policy, Duke University, Durham, NC 27708, USA; *Present address: Unidad de Biodiversidad y Conservación, Escuela Superior de Ciencias Experimentales y Tecnológicas, Universidad Rey Juan Carlos, c/Tulipán s/n, 28933 Móstoles, Spain

Author for correspondence:
Fernando T. Maestre
Tel: +1 919 6607414
Fax: +1 919 6607425
Email: maestre@duke.edu

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Summary

• Interactive effects of atmospheric CO_2 concentration ($[CO_2]$), soil nutrient availability and soil nutrient spatial distribution on the structure and function of plant assemblages remain largely unexplored.

• Here we conducted a microcosm experiment to evaluate these interactions using a grassland assemblage formed by *Lolium perenne*, *Plantago lanceolata*, *Trifolium repens*, *Anthoxanthum odoratum* and *Holcus lanatus*.

• Assemblages exhibited precise root foraging patterns, had higher total and below-ground biomass, and captured more nitrogen when nutrients were supplied heterogeneously. Root foraging responses were modified by nutrient availability, and the patterns of N capture by interactions between nutrient distribution, availability and $[CO_2]$. Greater above-ground biomass was observed under elevated CO_2 only under homogeneous conditions of nutrient supply and at the highest availability level. CO_2 interacted with nutrient distribution and availability to determine foliar percentage N and below : above-ground biomass ratios, respectively. Interactions between nutrient distribution and CO_2 determined the relative contribution to above-ground biomass of four of the species. The responses of dominant and subordinate species to $[CO_2]$ were dependent on the availability and distribution of nutrients.

• Our results suggest that soil nutrient distribution has the potential to influence the response of plant species and assemblages to changes in $[CO_2]$ and nutrient availability.

Key words: competition, elevated CO_2, microcosm, nitrogen (N) uptake, nutrient availability, soil nutrient heterogeneity.

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Introduction

Increasing concern about the ecological consequences of rising atmospheric CO_2 concentrations, a key component of global change (Houghton *et al.*, 2001), has boosted research to assess its effects on virtually all aspects of plant biology (Poorter, 1993; Wand *et al.*, 1999; Poorter & Navas, 2003). A substantial amount of this research has been conducted in controlled environments where plants have been grown under homogeneous soil conditions (Poorter, 1993). In the natural world, spatial heterogeneity in the availability of soil-based resources (hereafter termed soil nutrient heterogeneity) is the norm, rather than the exception, in most ecosystems (Jackson & Caldwell, 1993; Cain *et al.*, 1999; Farley & Fitter, 1999). At the spatial scale of the root system, soil nutrient heterogeneity promotes a suite of plant physiological and morphological responses, including changes in biomass allocation, root morphology, longevity and growth, and in

nutrient uptake patterns (Robinson, 1994; Huber-Sannwald & Jackson, 2001; Hodge, 2004). These responses determine the competitive ability and survival of individual plants within assemblages (Hodge, 2004), and thus soil nutrient heterogeneity has the potential to modify assemblage composition and productivity (Bliss *et al.*, 2002; Wijeshinge *et al.*, 2005; but see Casper & Cahill, 1996).

During the past decade, elevated CO_2 research has developed towards more complex experiments that evaluate the joint effects of elevated CO_2 and other environmental factors on plant performance and ecosystem processes (Stöcklin *et al.*, 1998; Zavaleta *et al.*, 2003; Reich *et al.*, 2004). These experiments have demonstrated that plant responses to elevated CO_2 at both species and assemblage levels are often dependent on the availability of soil nutrients such as nitrogen and phosphorus (Berntson & Bazzaz, 1997; Stöcklin *et al.*, 1998; Bassirirad *et al.*, 2001). However, it is unknown whether such responses are modified by other nutrient attributes, including their spatial distribution, because interactions between soil nutrient heterogeneity and elevated CO_2 on plant assemblages have barely begun to be explored (Arnone, 1997). At the species level, interactions between elevated CO_2 and soil nutrient heterogeneity may occur because soil nutrient heterogeneity promotes plant responses, such as changes in biomass allocation and nutrient uptake patterns, that are also a consequence of elevated CO_2 (Bassirirad *et al.*, 2001; Poorter & Navas, 2003; Hodge, 2004). At the assemblage level, such interactions may occur because co-occurring plants often differ in their ability to profit from this heterogeneity (Bliss *et al.*, 2002; Wijesinghe *et al.*, 2005), and in the direction and magnitude of their responses to elevated CO_2 (Berntson *et al.*, 1998; Grünzweig & Körner, 2001). Given the expectation that nutrients are usually distributed heterogeneously in soils, the potential for soil nutrient heterogeneity to interact with elevated CO_2 to determine plant species and assemblages responses is large.

To our knowledge, no previous study has evaluated the joint effects of elevated CO_2, overall soil nutrient availability and nutrient heterogeneity on plant performance, at either the species or assemblage level. To address this need, we conducted a microcosm experiment to evaluate the joint effects of these variables on the response of an assemblage formed by *Lolium perenne* L., *Plantago lanceolata* L., *Anthoxantum odoratum* L., *Holcus lanatus* L. and *Trifolium repens* L. These species commonly co-occur in seminatural temperate grasslands (Joshi *et al.*, 2000) and show differences in the magnitude of their responses to elevated CO_2, soil nutrient heterogeneity and nutrient availability (Staddon *et al.*, 1999; Goverde *et al.*, 2002; Hodge, 2004). We used natural soil and organic material (labeled with ^{15}N), rather than growing medium and inorganic fertilizer, because they are relevant materials that permit interpretation of plant responses to varying soil nutrient availability and heterogeneity in a more realistic context (Hodge, 2004). We tested the following hypotheses.

(i) The response of plant assemblage traits to soil nutrient heterogeneity, elevated CO_2 and nutrient availability will not be predictable from the assemblage responses to any one of these single factors. That is, statistical interactions between these factors will occur because soil nutrient heterogeneity and elevated CO_2 promote a similar suite of plant assemblage responses, and these responses are dependent on overall soil nutrient availability (Körner, 2003; Hodge, 2004).

(ii) Interactions between elevated CO_2, soil nutrient heterogeneity and overall soil nutrient availability will determine the species composition of the plant assemblage. These changes are predicted because of species-specific differences in traits related to their ability to respond to these factors.

Materials and Methods

Experimental design

We conducted a factorial microcosm experiment in the Duke University Phytotron between May and August 2004. The design of the experiment consisted of two levels of atmospheric CO_2 (37.5 and 70 Pa); three fertilization levels (40, 60 and 80 mg of N added as ^{15}N-labelled organic material); and two levels of spatial distribution of the organic material (homogeneous and heterogeneous), resulting in 12 treatment combinations. Microcosms consisted of PVC pipe (length 38 cm, internal diameter 10 cm) filled with, from the base, 5 cm of gravel (for drainage), then 28 cm of a mixture of soil and sand (50 : 50). The soil was a sandy loam of the White Store series and was collected from the top 30 cm of mineral horizon in the Duke Forest (35°55′ N, 78°52′ W). The resulting mix of soil and sand (hereafter 'background soil') had low fertility (3.70 ± 0.05 μg $N\text{-}NH_4$ g^{-1} soil and 0.27 ± 0.01 μg $N\text{-}NO_3$ g^{-1} soil; means ± SD, $n = 3$). On top of the background soil we placed a 2-cm layer of a 50 : 50 mixture of organic Duke Forest soil : peat to avoid the formation of physical crusts in the surface of the microcosms, and to simulate the typical accumulation of organic matter in the topsoil of temperate grasslands. All microcosms were irrigated with 200 ml of a soil microbial 'inoculum'. To obtain this inoculum, 3 kg fresh soil from turf communities (dominated by *T. repens*, *P. lanceolata* and *A. odoratum*) surrounding the Phytotron was mixed with 30 l water and the mixture agitated every 8 h for 2 d. The resulting solution was filtered with a 106 μm sieve and added to the microcosms before addition of the organic soil.

To the microcosms we added ^{15}N-labelled organic material which consisted of ground, oven-dried *L. perenne* shoots (5.30 at% ^{15}N, 2.91% N, 14.5 C : N). We generated this material by growing *L. perenne* in a 3 : 1 sand: vermiculite mix placed in a walk-in growth chamber that was maintained at day/night air temperatures of 21/16°C, PAR 1000 μmol $m^{-2}s^{-1}$ with a 16 h photoperiod, atmospheric partial pressure CO_2

37.5 Pa, and an average relative humidity of 80%. The growing medium was irrigated to free drainage with distilled water twice daily and received a modified half-strength Hoagland's solution containing 800 mg l^{-1} $^{15}NH_4{}^{15}NO_3$ (5 at% ^{15}N; Isotec, Miamisburg, OH, USA) twice weekly. After 8 wk growth, *L. perenne* plants were harvested and their shoots and roots dried at 60°C to constant weight.

To generate the three levels of soil nutrient availability we added different amounts of finely cut (to 2 mm lengths) ^{15}N-labelled *L. perenne* shoots. The amounts added were 1.38, 2.07 and 2.75 g, equivalent to 40, 60 and 80 mg N per microcosm, respectively. Within each nutrient availability level, the organic material was added homogeneously (homogeneous treatment) or as a patch (heterogeneous treatment). In the homogeneous treatment we mixed the organic material with the background soil before introducing it into the PVC pipe. In the heterogeneous treatment, the organic material was localized within discrete 31 cm^3 volumes of soil. To create one of these patches, we mixed 25 cm^3 background soil with the organic material and introduced the resulting mix into a 31-cm^3 plastic cylinder (length 75 mm; internal diameter 23 mm) consisting of a light mesh with square pores 5 × 10 mm in size. We refer to this as the patch cylinder. A second (control) cylinder, filled only with background soil, was placed 2 cm apart and parallel to the patch cylinder. Cylinders were located 12 cm below the surface of the organic soil. The homogeneous treatment received two plastic control cylinders only.

Seeds from the five species, obtained from commercial suppliers, were placed in trays with plant growing medium (Metro-Mix 200, Scotts Co., Marysville, OH, USA) and germinated in a growth chamber (20°C, PAR 500 µmol $m^{-2}s^{-1}$, 14 h photoperiod). Because of different germination times, species were germinated on different days to ensure all species were of similar size (two-leaf stage) at the start of the experiment. On 10 May 2004, two seedlings of each species were transplanted into each microcosm. The planting pattern was allocated once at random and adhered to for all microcosms. Seedlings that died during the first week of the experiment were replaced; no further mortality was observed after this time.

We established six microcosms for each of the 12 treatment combinations, providing 72 assemblages total (two CO_2 levels × three nutrient availability levels × two nutrient heterogeneity levels × six replicates). These assemblages were placed in four walk-in growth chambers (two for each CO_2 level) within which climate was independently controlled. For each CO_2 level, three replicates per treatment were assigned randomly to one of the chambers. The positions of the microcosms within each chamber were randomly interchanged each week. Temperatures in the growth chambers ranged from 12°C at night to 21°C during the day, and this regime included a simulated dawn and dusk period, each of 2 h duration, where temperature was gradually ramped up or down. Relative humidity followed a similar pattern, ranging from 85 to 70%, as did the lights. The maximum PAR in the 15-h photoperiod was 900 µmol $m^{-2}s^{-1}$.

Microcosms were irrigated twice a day with 50 ml distilled water during the first 2 wk of the experiment, and once a day with the same amount thereafter. To promote the formation of nodules on *T. repens* roots, all microcosms were watered with 50 ml of a 106-µm sieved solution derived from root macerations (roots were collected from the turf communities surrounding the Phytotron). To reduce limitations to plant growth caused by low overall soil fertility, all microcosms received 50 ml of a nutrient solution containing 35 mg calcium (added as $CaCl_2$ $2H_2O$) and 29 mg magnesium (added as $MgSO_4{\cdot}7H_2O$) five times during the course of the experiment (12 May, 8 June, 22 June, 6 July and 13 July).

Harvest

Plants were grown in the chambers for 90 d. After this time, the above-ground biomass of all the microcosms was cut at the soil surface and sorted to species. Leaves and stems were dried at 60°C until constant weight. After above-ground harvesting, soil was carefully removed from a microcosm unit and the roots harvested. They were not sorted to species. To measure root proliferation, we harvested the roots within control and patch cylinders separately from the bulk soil.

Root foraging precision was estimated with the index RII (Armas *et al.*, 2004). In the heterogeneous treatments, RII was calculated as $(RB_p - RB_c)/(RB_p + RB_c)$, where RB_p and RB_c are the root biomass in the patch and control cylinders, respectively. RII ranges from −1 to +1: a value of zero indicates equal root growth in nutrient patches and background soil (no precision of foraging). Increasing positive values indicate increasing precision (root proliferation into the nutrient patch). In the homogeneous treatment the control cylinder, in the same location as the patch cylinder in the heterogeneous treatment, was treated as the patch cylinder for the purposes of calculating the RII index.

From the species and assemblage biomass data we calculated the WinRatio index (Poorter & Navas, 2003). For a given combination of nutrient heterogeneity and availability, it is estimated as $(BS_e - BS_a)/(BA_e - BA_a)$, where BS_e and BS_a are the average biomass of the species at elevated and ambient CO_2, respectively, and BA_e and BA_a are the average biomass of the assemblage at elevated and ambient CO_2, respectively. If this ratio is >1, the species is profiting disproportionally from elevated CO_2 [a 'winner' *sensu* Poorter & Navas (2003)]. If the ratio is <1, the species is 'losing out' relative to the assemblage as a whole.

Measurement of nitrogen captured from added organic material

Uptake of N from added organic material was determined by species for the foliar material and by whole assemblage for the

roots. Roots and leaves were ground to a fine powder, and a subsample was injected into an elemental analyser (Costech CHN Analyser, Milan, Italy) coupled to an isotope ratio mass spectrometer (Finnigan Delta Plus, Bremen, Germany) via a Finnigan Conflo Interface. The at% ^{15}N excess was calculated by subtracting 0.366 (atmospheric background). The amount of N captured was estimated as the percentage of N added in the organic material that was captured by a species {[(mg ^{15}N in foliar tissue)/(mg ^{15}N in added organic material)] × 100} or the assemblage {[(sum of mg ^{15}N obtained in roots and leaves of each species)/(mg ^{15}N in added organic material)] × 100} (Hodge *et al.*, 2000).

Statistical analyses

The effects of treatments on assemblage properties were analysed separately for each variable using a four-way nested ANOVA model. The model included CO_2, nutrient heterogeneity and nutrient availability as main fixed factors; growth chamber as a random factor nested within CO_2; and all possible interactions between the fixed factors. In this model, the effect of CO_2 (1 df) was tested against the random effect of chamber nested within CO_2 (2 df). The main effects of nutrient heterogeneity (1 df) and availability (2 df), as well as the two- and three-factor interactions, were tested against the residual error (58 df). To control for differences in plant size when evaluating the patterns of biomass allocation (Reich, 2002), we analyzed not the below : above-ground ratio, but instead the residuals from a regression between the below : above-ground ratio (dependent variable) and the total biomass data (independent variable). Species composition was expressed as the percentage of total biomass accounted for by each species (see Figure S1, available online as supplementary material, for the original biomass data). Data obtained at the individual species level were analysed separately for each species using the four-way nested ANOVA model described above. Despite limitations (Anderson, 2001), this model is routinely used because it facilitates ready interpretation of the effects of different factors on the performance of species within an assemblage (Berntson *et al.*, 1998; Grünzweig & Körner, 2001; Joel *et al.*, 2001). Where appropriate, Tukey's *B*-test was used for *post hoc* comparisons.

Relationships between root foraging precision and total biomass of assemblages, and between the effects of elevated CO_2 on N uptake and net biomass production, were evaluated using linear regression. Mean values of each nutrient heterogeneity and availability treatment were considered as the units of observation. Data were log-transformed where necessary to meet the assumptions of ANOVA. Analyses were performed using SPSS ver. 10.0 (SPSS Inc., Chicago, IL, USA). Although we conducted a large number of statistical tests, given the ANOVA design we used, *P* values were not adjusted for multiple testing as this approach is considered overly conservative (Gotelli & Ellison, 2004).

Results

Responses at assemblage level

Total and below-ground biomass were significantly affected by nutrient heterogeneity and availability (Fig. 1; Table S1, available online as supplementary material). For both response variables, higher biomass was achieved under heterogeneous conditions and under the highest nutrient availability ($P < 0.001$ in both cases, no significant interactions). For above-ground biomass, significant CO_2 × nutrient heterogeneity ($P = 0.036$) and CO_2 × nutrient availability ($P = 0.013$) interactions were found (Table S1). We investigated the CO_2 × nutrient heterogeneity interaction by conducting ANOVAs for each heterogeneity level. These revealed significant effects of both CO_2 ($F_{1,30} = 20$, $P < 0.001$) and nutrient availability ($F_{2,30} = 12$, $P < 0.001$) on above-ground biomass under homogeneous conditions, with assemblages having more above-ground biomass under elevated CO_2 and at the highest nutrient availability (Fig. 1). Under heterogeneous conditions, no significant effects of CO_2 were detected, but there were significant differences between the levels of nutrient availability ($F_{2,30} = 20$, $P < 0.001$; *post hoc* results not shown). We next investigated the CO_2 × nutrient availability interaction. This revealed nonsignificant ($F_{1,20} = 0.45$, $P = 0.509$) and marginally significant ($F_{1,20} = 4.0$, $P = 0.060$) effects of CO_2 and soil nutrient heterogeneity, respectively, at the lowest nutrient availability level. In contrast, the effects of both factors were significant at the medium (CO_2: $F_{1,20} = 8.9$, $P = 0.007$; nutrient heterogeneity: $F_{1,20} = 6.6$, $P = 0.019$) and highest (CO_2: $F_{1,20} = 13$, $P = 0.002$; nutrient heterogeneity: $F_{1,20} = 6.4$, $P = 0.020$) nutrient availability levels, with assemblages having more above-ground biomass under heterogeneous and under elevated CO_2 conditions (Fig. 1).

A significant CO_2 × nutrient availability interaction ($P = 0.007$) was found for the below : above-ground biomass ratio (Fig. 1; Table S1). Investigation of this interaction revealed that the effects of CO_2 were not significant at the lowest nutrient availability level ($F_{1,20} = 0.12$, $P = 0.731$), but were significant at the medium ($F_{1,20} = 31.36$, $P < 0.001$) and highest ($F_{1,20} = 5.87$, $P = 0.025$) levels. In the latter two cases, the below : above-ground biomass ratio was lower under elevated CO_2 conditions. The effect of soil nutrient heterogeneity was not significant at either nutrient availability level ($P > 0.440$ in all cases).

The concentration of N in below-ground tissues was significantly less in the heterogeneous relative to the homogeneous treatment (Table 1; Table S1). A significant CO_2 × nutrient heterogeneity interaction ($P = 0.041$) was found for the N concentration in above-ground tissues (Table S1). Investigation of this interaction revealed that, under homogeneous conditions, assemblages had lower N concentrations under elevated CO_2 ($F_{1,30} = 5.1$, $P = 0.031$) but no such effects were found under heterogeneous conditions ($P > 0.520$; Table 1). Nutrient availability did not significantly affect this variable. A marginally significant CO_2 × nutrient availability interaction ($P = 0.092$)

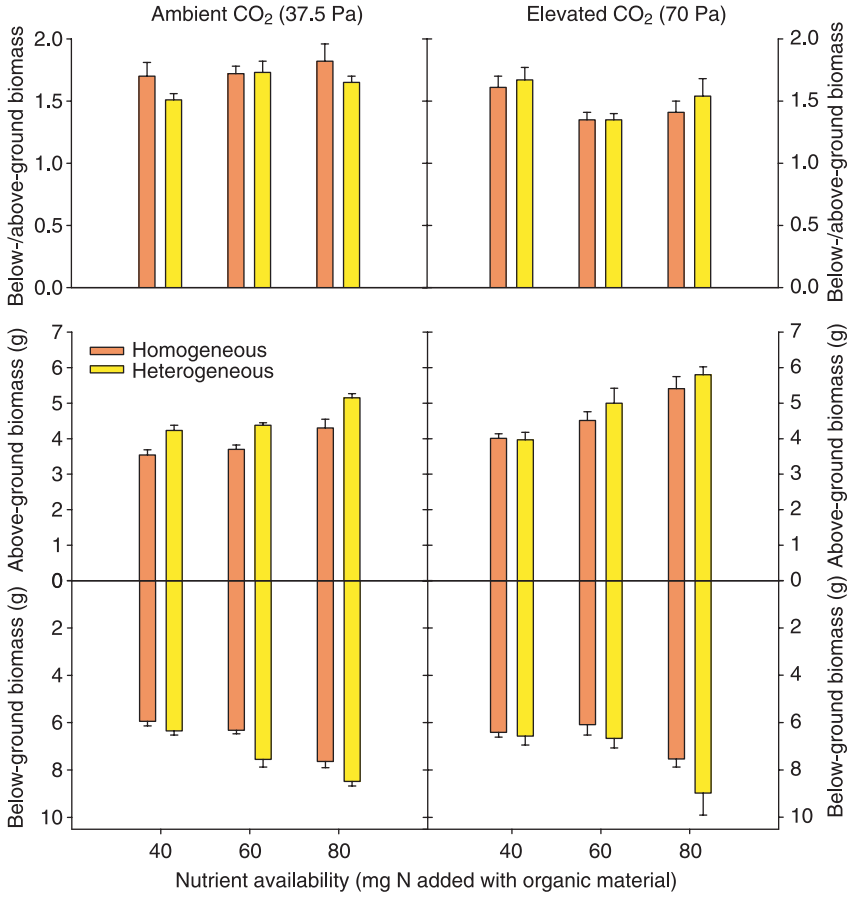

Fig. 1 Below : above-ground biomass ratio (upper panels); above-ground biomass (central panels); and below-ground biomass (lower panels) of the assemblages (formed by *Lolium perenne*, *Plantago lanceolata*, *Trifolium repens*, *Anthoxanthum odoratum* and *Holcus lanatus*) at the end of the experiment. Values are means ± 1 SE (n = 6). See Table S1 for statistical analysis.

Table 1 Nitrogen concentration of, and capture by, the assemblages (formed by *Lolium perenne*, *Plantago lanceolata*, *Trifolium repens*, *Anthoxanthum odoratum* and *Holcus lanatus*)

CO_2 (Pa)	NH	NA (mg N)	ANC (%)	BNC (%)	TNC (mg)	NCA (%)	ANB (%)
37.5	HOM	40	0.74 ± 0.06	0.69 ± 0.05	66.13 ± 2.62	19.65 ± 0.70	88.00 ± 0.91
		60	0.77 ± 0.05	0.63 ± 0.03	67.99 ± 3.55	22.57 ± 0.93	80.05 ± 0.98
		80	0.73 ± 0.04	0.60 ± 0.03	76.37 ± 3.28	19.61 ± 1.37	79.44 ± 1.65
	HET	40	0.64 ± 0.04	0.59 ± 0.03	63.36 ± 2.79	27.25 ± 0.91	82.71 ± 1.00
		60	0.65 ± 0.05	0.57 ± 0.03	69.37 ± 4.69	26.14 ± 1.27	77.15 ± 1.53
		80	0.68 ± 0.08	0.57 ± 0.02	79.72 ± 4.55	27.95 ± 1.76	72.20 ± 0.76
70	HOM	40	0.63 ± 0.04	0.62 ± 0.06	63.87 ± 3.56	20.41 ± 0.53	87.17 ± 0.50
		60	0.66 ± 0.07	0.66 ± 0.02	68.79 ± 3.18	18.05 ± 0.88	84.28 ± 0.69
		80	0.66 ± 0.04	0.66 ± 0.03	83.82 ± 2.10	20.69 ± 0.67	80.42 ± 0.64
	HET	40	0.67 ± 0.04	0.60 ± 0.02	63.61 ± 1.06	24.27 ± 1.11	84.84 ± 0.56
		60	0.64 ± 0.06	0.62 ± 0.03	69.72 ± 1.70	25.36 ± 1.59	78.36 ± 1.09
		80	0.72 ± 0.05	0.58 ± 0.04	90.29 ± 4.38	28.22 ± 0.96	75.10 ± 0.87

Values are means ± 1 SE, n = 6.
CO_2, Atmospheric concentration of CO_2; NH, nutrient heterogeneity; NA, nutrient availability; ANC, N concentration in above-ground tissues; BNC, N concentration in below-ground tissues; TNC, total N content of assemblages (roots plus shoots); NCA, percentage of N added with organic material captured by assemblages (roots plus shoots); ANB, percentage of total N captured by assemblages derived from background soil (roots plus shoots); HOM, homogeneous treatment; HET, heterogeneous treatment. See Table S1 for statistical analyses of these data.

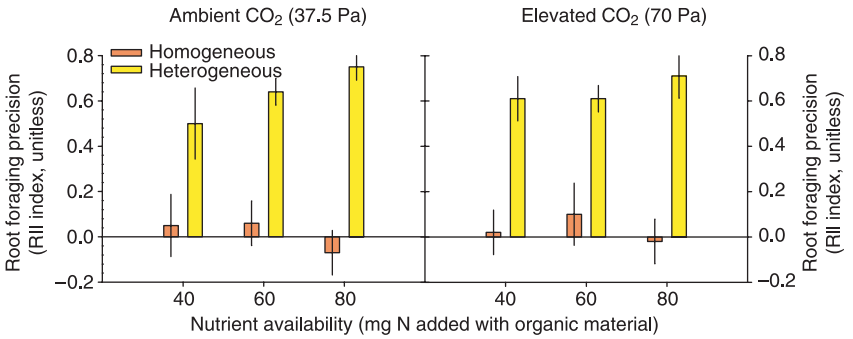

Fig. 2 Root foraging precision into nutrient patches. Positive values of the RII index indicate precise root foraging (see Materials and Methods for details on its calculation). Values are means ± 95% confidence intervals (n = 6). Significant root foraging patterns are indicated by confidence intervals that do not overlap 0. See Table S1 for statistical analysis.

was found for the total N content of the assemblages (Table 1; Table S1). Investigation of this interaction revealed that the effects of CO_2 were not significant at the lowest ($F_{1,20}$ = 0.14, P = 0.715) and medium ($F_{1,20}$ = 0.07, P = 0.791) nutrient availability levels, but were significant at the highest ($F_{1,20}$ = 6.4, P = 0.020) level. In the latter case, the total N content of the assemblages (including roots and shoots) was higher under elevated relative to ambient CO_2 conditions. Soil nutrient heterogeneity had no significant (P > 0.225 in all cases) effect on this variable.

A marginally significant (P = 0.059) three-way interaction was found for the proportion of N captured from the added organic material (NCA, Table 1; Table S1). Investigation of this interaction by CO_2 level revealed that the effect of nutrient heterogeneity was significant at both ambient ($F_{1,30}$ = 43, P < 0.001) and elevated CO_2 ($F_{1,30}$ = 57, P < 0.001), with assemblages capturing more N when the organic material was added as a patch (Table 1). A significant effect of soil nutrient availability was found only under elevated CO_2 ($F_{2,30}$ = 4.0, P = 0.028). At this CO_2 concentration, assemblages captured more N at the highest compared with the lowest nutrient availability level (*post hoc* results not shown; Table 1).

A significant (P = 0.045) three-way interaction was found for the proportion of total N captured by the assemblage that derived from the background soil (ANB, Table 1; Table S1). Note that ANB and NCA are not the inverse of one another, as they reflect the proportion of N captured by the assemblage that was added and the proportion of the total N content of the assemblages that is derived from the background soil, respectively. Investigation of this interaction by CO_2 level revealed significant effects of both soil nutrient heterogeneity ($F_{1,30}$ = 29, P < 0.001) and availability ($F_{2,30}$ = 34, P < 0.001) at ambient CO_2, with assemblages capturing less N from the background soil under heterogeneous conditions and with decreasing nutrient availability (*post hoc* results not shown; Table 1). A similar pattern was observed at elevated CO_2 (Table 1), but in this case a marginally significant interaction between nutrient heterogeneity and availability was found ($F_{2,30}$ = 3.27, P = 0.052).

When the organic material was supplied heterogeneously, the assemblages demonstrated precise root foraging patterns (Fig. 2). The magnitude of the differences in root foraging precision between homogeneous and heterogeneous treatments was affected by nutrient availability (heterogeneity × nutrient availability interaction: P = 0.001, Table S1). Investigation of this interaction revealed that, as expected, precision in root foraging did not vary with nutrient availability in the homogeneous treatment. However in the heterogeneous treatment there was a significant effect of availability ($F_{2,28}$ = 6.7, P = 0.004), with higher proliferation in the highest compared with the lowest nutrient availability (P < 0.05, *post hoc* results not shown; Fig. 2). Elevated CO_2 had no significant effects on root proliferation, nor did it modify the effects of nutrient availability and heterogeneity on foraging precision patterns. In the heterogeneous treatment, root foraging precision was positively related to total biomass (Fig. 3).

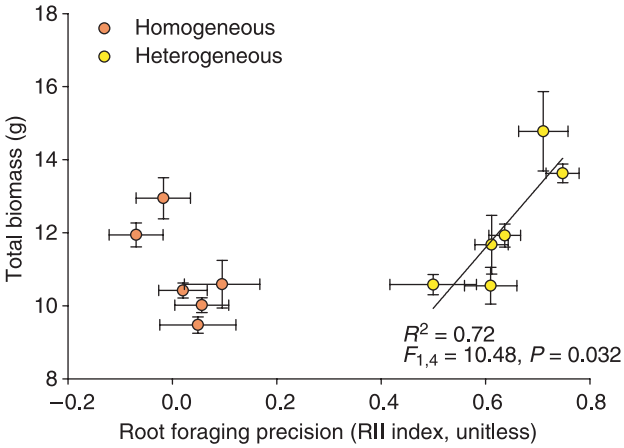

Fig. 3 Relationship between root foraging precision and total biomass of the assemblages (formed by *Lolium perenne*, *Plantago lanceolata*, *Trifolium repens*, *Anthoxanthum odoratum* and *Holcus lanatus*). Separate regression analyses were conducted for data from the homogeneous and heterogeneous treatments; only significant regression relationships are shown. Each point represents the average value for a given combination of nutrient availability and CO_2 concentration. Values are means ± 1 SE (n = 6).

Responses at species level

Holcus lanatus was the dominant species in the assemblages, accounting for >60% of the mean total above-ground biomass

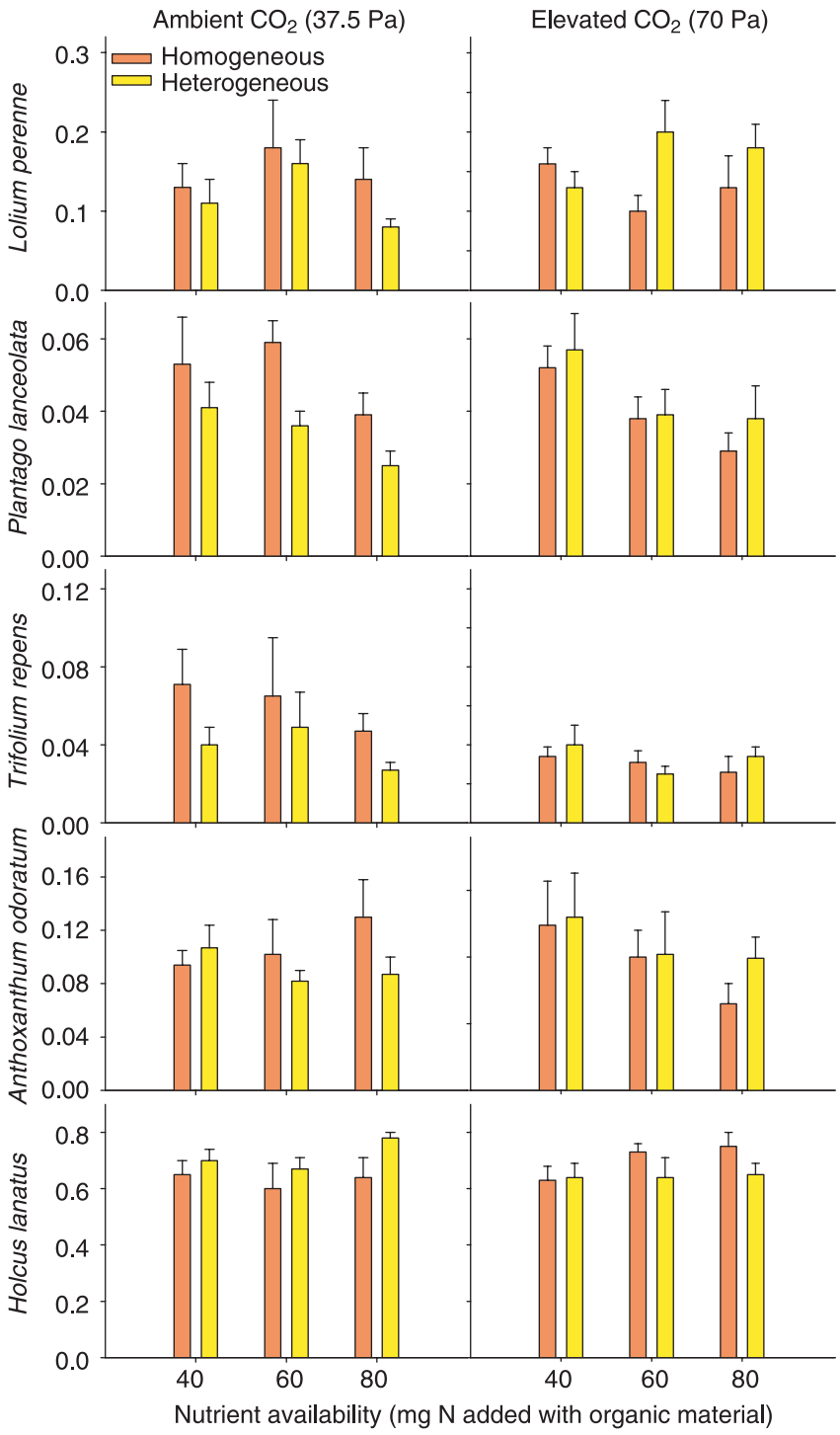

Fig. 4 Proportion of total above-ground biomass accounted for by each of the different species in the assemblages. Values are means ± 1 SE (n = 6). Note difference in scale of y axes. See Table S2 for statistical analysis and Figure S1 for raw biomass values.

in all treatment combinations (Fig. 4). *Lolium perenne* and *A. odoratum* were, in order, the next most dominant; they had proportions ranging from 8 to 20% and from 7 to 14%, respectively, of the total above-ground biomass. Biomass of *T. repens* and *P. lanceolata* was always <8% of the above-ground total. The proportion of total above-ground biomass accounted for by *A. adoratum* was not significantly affected by treatment (Table S2, available online as supplementary material; Fig. 4). A significant $CO_2 \times$ nutrient heterogeneity interaction was found for *P. lanceolata* (P = 0.018) and *H. lanatus* (P = 0.015), and a marked one for *L. perenne* (P = 0.058) and *T. repens* (P = 0.060) (Table S2). Investigation of these interactions by CO_2 level revealed that, at ambient CO_2, the proportion of above-ground biomass accounted for by *P. lanceolata* was lower

under heterogeneous conditions ($F_{2,30}$ = 6.8, P = 0.014), but that accounted for by *H. lanatus* was greater ($F_{2,30}$ = 3.5, P = 0.071; Fig. 4). No significant heterogeneity effects were observed for either species at elevated CO_2 (Fig. 4). The analysis of these interactions in the case of *Trifolium* and *Lolium* gave no significant effects of nutrient heterogeneity and availability at any CO_2 level. *Plantago lanceolata* also responded significantly (P = 0.006) to nutrient availability, having lower proportions under the highest as compared with the lowest nutrient availability (P < 0.05, *post hoc* results not shown; Fig. 4).

The results of the WinRatio index (Fig. 5) demonstrated a clear effect of both soil nutrient heterogeneity and availability on the biomass responses of the different species to elevated CO_2. Under homogeneous conditions, *L. perenne*, *P. lanceolata* and *A. odoratum* were 'winner' species under the lowest nutrient availability, and *H. lanatus* only was the 'winner' under the medium and highest nutrient availability levels. This pattern was different under heterogeneous conditions: *H. lanatus* was never a 'winner' and *L. perenne*, *P. lanceolata* and *A. odoratum* were winners under all nutrient availability levels (*T. repens* was also a 'winner' at the highest nutrient availability level; Fig. 5).

The foliar N concentrations and C : N ratios of the species were not affected by the treatments (Table 2; Tables S3 and S4, available online as supplementary material). In contrast, there were treatment effects on the uptake of N from the added organic material, but these effects were species-dependent (Table 2; Table S5, available online as supplementary material). Nitrogen uptake patterns in *P. lanceolata* were not affected by the treatments. A marginally significant (P = 0.092) three-way interaction was found for *T. repens*. Investigation of this interaction by CO_2 level revealed no significant effects of both nutrient heterogeneity and availability at either CO_2 level. *Anthoxanthum odoratum* captured significantly more N when the organic material was added as a patch (P = 0.006). A significant CO_2 × nutrient heterogeneity interaction was found for *H. lanatus* and *L. perenne*. Investigation of these interactions by CO_2 level revealed that, at ambient CO_2, the capture of N from the organic material was greater under heterogeneous conditions for *H. lanatus* ($F_{1,30}$ = 20, P < 0.001), but was not affected by heterogeneity for *L. perenne* ($F_{1,30}$ = 1.1, P = 0.310). The opposite pattern was observed at elevated CO_2: more N was captured under heterogeneous conditions by *L. perenne* ($F_{1,30}$ = 7.5, P = 0.010), whereas capture by *H. lanatus* was unaffected by heterogeneity ($F_{1,30}$ = 2.4, P = 0.129; Table 2). When data from across all species were combined, the enhancement in N uptake at elevated relative to ambient CO_2 levels was positively related to that in above-ground biomass production (Fig. 6).

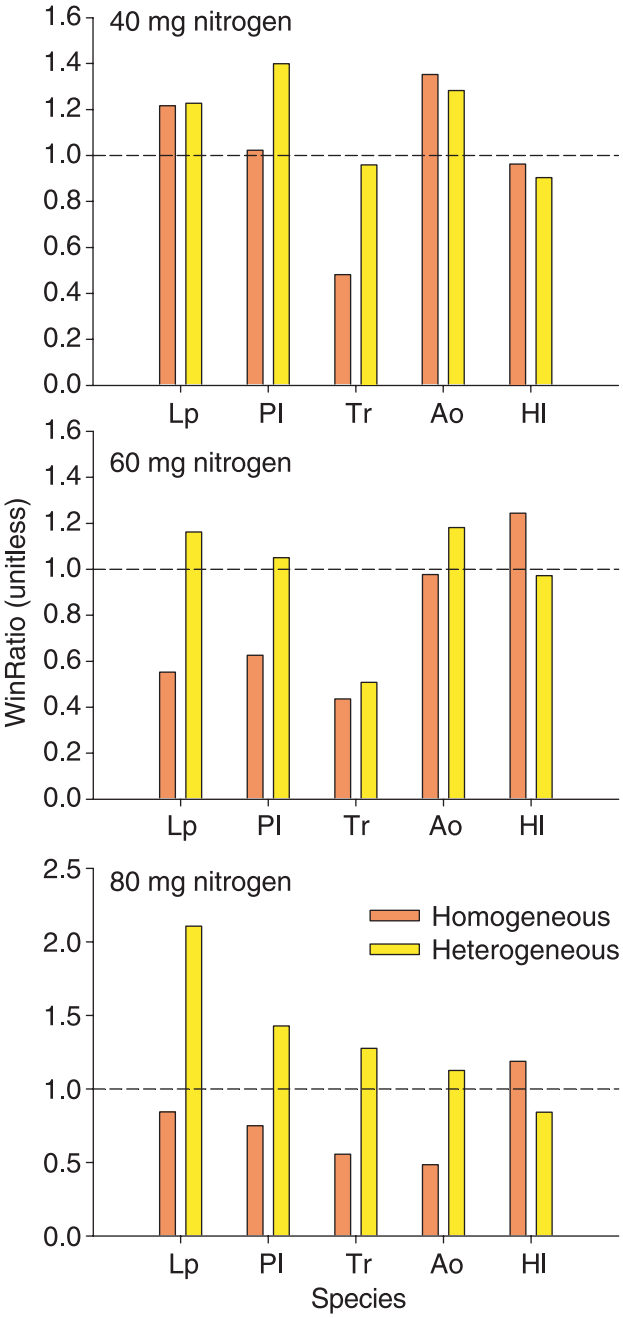

Fig. 5 Biomass enhancement ratio (BER) value of each species divided by the BER of the assemblage in the different treatments (WinRatio). BER values were obtained by dividing the average above-ground biomass obtained at 70 Pa CO_2 by that obtained at 37.5 Pa. Lp, *Lolium perenne*; Pl, *Plantago lanceolata*; Tr, *Trifolium repens*; Ao, *Anthoxanthum odoratum*; Hl, *Holcus lanatus*.

Discussion

We found interactive effects of elevated CO_2 and soil nutrient heterogeneity or availability, respectively, on a number of assemblage characteristics (biomass, foliar N concentration, root foraging responses). Notably, the only responses that were consistent with our first hypothesis (three-way interactions between nutrient heterogeneity, CO_2 and nutrient availability) were observed for variables related to the patterns of N uptake by the assemblages. The fact that N uptake patterns were

Table 2 Nitrogen concentration (ANC), carbon:nitrogen ratio (C:N) and percentage of N initially added with organic material captured (NCA) by each species

Species	CO_2 (Pa)	NH	NA (mg N)	ANC (%)	C : N	NCA (%)
Lolium perenne	37.5	HOM	40	0.91 ± 0.07	46.70 ± 3.65	2.00 ± 0.49
			60	0.88 ± 0.05	48.04 ± 3.04	2.83 ± 1.02
			80	0.87 ± 0.09	48.72 ± 4.10	2.02 ± 0.82
		HET	40	0.79 ± 0.10	49.99 ± 3.83	1.35 ± 0.36
			60	0.69 ± 0.06	62.48 ± 6.73	2.16 ± 0.50
			80	0.88 ± 0.03	47.20 ± 1.84	1.66 ± 0.53
	70	HOM	40	0.78 ± 0.05	54.40 ± 3.23	2.21 ± 0.38
			60	0.83 ± 0.06	51.49 ± 2.91	1.48 ± 0.45
			80	0.90 ± 0.12	50.46 ± 5.23	2.16 ± 0.75
		HET	40	0.88 ± 0.14	51.68 ± 5.17	2.07 ± 0.49
			60	0.76 ± 0.05	56.72 ± 3.91	4.17 ± 1.12
			80	0.89 ± 0.09	49.80 ± 4.87	4.70 ± 1.03
Plantago lanceolata	37.5	HOM	40	0.69 ± 0.08	63.03 ± 9.62	0.35 ± 0.10
			60	0.66 ± 0.04	63.23 ± 3.31	0.49 ± 0.14
			80	0.65 ± 0.05	64.81 ± 4.12	0.23 ± 0.09
		HET	40	0.69 ± 0.05	66.83 ± 5.02	0.42 ± 0.22
			60	0.65 ± 0.02	64.98 ± 3.01	0.40 ± 0.15
			80	0.77 ± 0.09	57.49 ± 5.64	0.17 ± 0.04
	70	HOM	40	0.73 ± 0.07	61.07 ± 6.00	0.50 ± 0.10
			60	0.61 ± 0.06	73.39 ± 8.25	0.19 ± 0.05
			80	0.73 ± 0.04	58.84 ± 2.96	0.17 ± 0.03
		HET	40	0.74 ± 0.07	60.41 ± 6.06	0.61 ± 0.25
			60	0.77 ± 0.05	56.37 ± 4.04	0.49 ± 0.13
			80	0.81 ± 0.05	52.58 ± 3.28	0.61 ± 0.25
Trifolium repens	37.5	HOM	40	1.76 ± 0.29	26.84 ± 6.32	0.11 ± 0.03
			60	1.87 ± 0.16	22.95 ± 1.88	0.09 ± 0.05
			80	1.99 ± 0.12	22.03 ± 1.13	0.08 ± 0.01
		HET	40	2.04 ± 0.18	21.33 ± 1.82	0.23 ± 0.07
			60	1.88 ± 0.05	22.22 ± 0.64	0.26 ± 0.12
			80	1.87 ± 0.10	22.71 ± 1.32	0.15 ± 0.06
	70	HOM	40	1.95 ± 0.24	22.93 ± 2.41	0.09 ± 0.03
			60	2.00 ± 0.19	22.41 ± 1.90	0.06 ± 0.02
			80	2.34 ± 0.34	19.67 ± 2.12	0.07 ± 0.05
		HET	40	2.11 ± 0.21	20.29 ± 1.96	0.18 ± 0.06
			60	2.07 ± 0.14	20.65 ± 1.38	0.03 ± 0.01
			80	1.74 ± 0.12	24.91 ± 1.65	0.43 ± 0.26
Anthoxanthum odoratum	37.5	HOM	40	0.72 ± 0.07	54.71 ± 2.67	0.35 ± 0.07
			60	0.66 ± 0.06	64.82 ± 8.07	0.30 ± 0.11
			80	0.77 ± 0.12	60.61 ± 9.44	0.63 ± 0.16
		HET	40	0.61 ± 0.08	75.02 ± 14.65	1.18 ± 0.54
			60	0.57 ± 0.05	73.51 ± 7.70	0.28 ± 0.05
			80	0.66 ± 0.02	62.53 ± 2.26	1.02 ± 0.22
	70	HOM	40	0.54 ± 0.05	78.38 ± 7.49	0.49 ± 0.21
			60	0.72 ± 0.05	58.89 ± 4.10	0.37 ± 0.08
			80	0.68 ± 0.06	61.88 ± 4.96	0.28 ± 0.09
		HET	40	0.64 ± 0.07	68.27 ± 8.22	1.23 ± 0.40
			60	0.65 ± 0.09	68.34 ± 9.47	1.10 ± 0.72
			80	0.64 ± 0.09	70.36 ± 8.66	1.01 ± 0.25
Holcus lanatus	37.5	HOM	40	0.58 ± 0.05	73.18 ± 6.71	5.70 ± 0.98
			60	0.66 ± 0.04	63.46 ± 5.17	5.98 ± 1.11
			80	0.62 ± 0.03	66.74 ± 3.63	6.02 ± 1.10
		HET	40	0.57 ± 0.05	73.62 ± 5.96	10.01 ± 0.66
			60	0.56 ± 0.04	73.47 ± 5.42	8.71 ± 1.12
			80	0.62 ± 0.10	77.60 ± 5.96	11.62 ± 1.69
	70	HET	40	0.53 ± 0.03	79.25 ± 3.70	5.37 ± 0.56
			60	0.58 ± 0.08	75.73 ± 8.75	6.25 ± 0.55
			80	0.53 ± 0.06	82.05 ± 7.90	6.79 ± 0.96
		HET	40	0.54 ± 0.05	79.65 ± 7.03	6.47 ± 0.91
			60	0.52 ± 0.06	84.93 ± 10.99	7.21 ± 1.47
			80	0.62 ± 0.04	68.88 ± 5.02	8.23 ± 0.71

Values are means ± 1 SE, $n = 6$.
CO_2, Atmospheric concentration of CO_2; NH, nutrient heterogeneity; NA, nutrient availability; HOM, homogeneous treatment; HET, heterogeneous treatment. See Tables S3–S5 for the statistical analysis of these data.

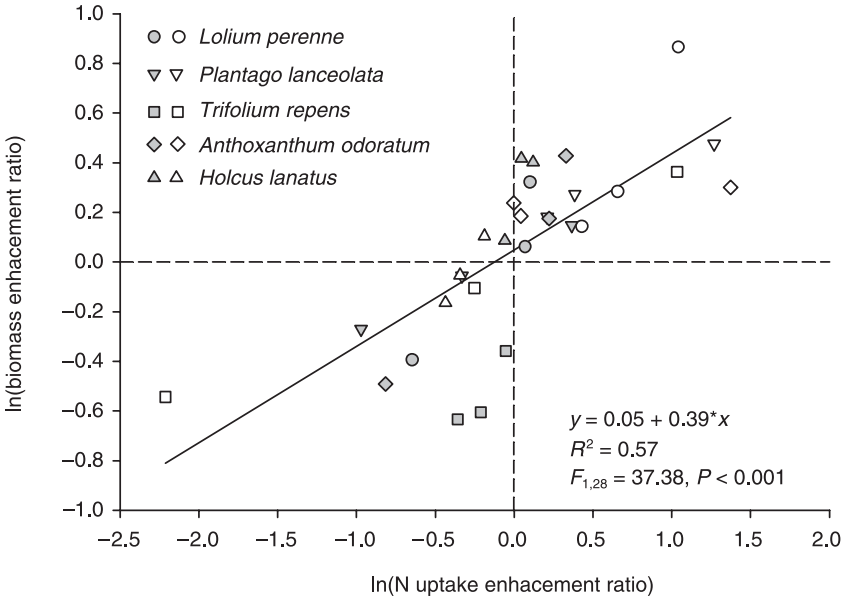

Fig. 6 Relationship between nitrogen uptake enhancement ratio (NER) and biomass enhancement ratio (BER) for the different species. For a given species and combination of soil nutrient heterogeneity and availability treatments, BER and NER values were obtained by dividing the average above-ground biomass and proportion of N captured from the organic material added obtained at 70 Pa CO_2 by those obtained at 37.5 Pa CO_2, respectively. For clarity of presentation, different nutrient availability levels are not shown. Grey symbols, homogeneous treatments; white symbols, heterogeneous treatments.

affected cautions that the responses of plant assemblages to joint increases in the concentration of CO_2 and in nutrient availability are likely to be influenced by nutrient heterogeneity. If the interactions we found stand in field ecosystems, then predicting responses of plant assemblages to elevated CO_2 by considering only observed responses will not be informative (Körner, 2003). Indeed, such responses will be an outcome of both interactive effects of other global change drivers, and interactions between these drivers and intrinsic ecosystem features such as soil nutrient heterogeneity.

As in previous studies conducted with grassland species (Stöcklin *et al.*, 1998; He *et al.*, 2002; Grünzweig & Körner, 2003), significant interactions between CO_2 and nutrient availability were found for assemblage attributes such as above-ground biomass, below : above-ground ratio and the amount of N captured from the added organic material. The increase in nutrient availability increased growth and N capture responses to elevated CO_2, suggesting that CO_2 effects on these assemblage attributes were constrained by low nutrient availability. To our knowledge, this study is the first to report a $CO_2 \times$ nutrient heterogeneity interaction when estimating plant performance using variables such as biomass and N content. In the only previous study that evaluated the joint effects of soil nutrient heterogeneity and CO_2 on plant performance, Arnone (1997) found that precision of root foraging measured with fine root length (his response variable) increased under elevated CO_2 69 d after the addition of the nutrient patches. Such a response was, however, not observed in our study, as indicated by the lack of a significant $CO_2 \times$ nutrient heterogeneity interaction when analysing precision in root foraging data. Notably, the positive effects of elevated CO_2 on above-ground biomass were observed only under homogeneous conditions. Thus our results suggest that benefits that plants obtain when growing in a CO_2-enriched atmosphere, such as reduced water requirements, and increases in soil water availability and N mineralization (Poorter, 1993; Niklaus & Körner, 2004), do not interact additively with those that plants obtain from soil nutrient heterogeneity (an increase in nutrient uptake resulting from precise root foraging into nutrient patches). More studies are needed to evaluate the generality of our findings, and to test if similar responses are observed with species belonging to other functional and community types.

Soil nutrient heterogeneity interacted with nutrient availability to determine root foraging responses. Under heterogeneous conditions, the proliferation of roots into the nutrient patches by the assemblages was greater with higher nutrient availability. The increasing contrast between the nutrient patch and background soil, a necessary result of increasing the amount of nutrients in a patch, potentially stimulated this proliferation. The contrast in nutrient content between a nutrient patch and the background soil is an important feature of heterogeneous soil environments that has received little attention to date (Hodge, 2004). If the nutrient demand in the background soil is not met, root proliferation in the nutrient patches should increase with increasing contrast between these patches and the background soil (Lamb *et al.*, 2004). However, previous studies conducted with single species suggest that the increase in root proliferation with increasing contrast between the patch and the background soil becomes saturated at high contrast levels (Wijesinghe & Hutchings, 1999; Lamb *et al.*, 2004). As discussed by Hodge (2004), root proliferation responses are only relevant for plant performance when different plant species compete for organic patches containing a limited local supply of different N sources, as such responses are critical to determine their competitive

success. Thus, in a competitive setting such as that evaluated here, we would expect that: (a) root proliferation by the assemblages under heterogeneous conditions increases with increasing contrast between the patch and the background soil; and (b) a saturating response of root proliferation by the assemblages, if any, should occur at higher contrast levels than those promoting a saturating response in species grown individually. However, our results do not provide definitive evidence for this affirmation, which should be tested in future studies.

Consistent with the findings of Day *et al.* (2003) and Wijesinghe *et al.* (2005) (but see Casper & Cahill, 1996), higher assemblage biomass was observed when nutrients were supplied heterogeneously (Fig. 1). These biomass responses were associated with precise root foraging in response to heterogeneous nutrient supply (Fig. 2), suggesting that root foraging precision is a key driver of the assemblage biomass responses. This assertion appears to be supported by the fact the assemblages captured more N from the organic material added when it was supplied heterogeneously (Table 1), and their total biomass increased linearly with increased foraging precision under these conditions (Fig. 3). Physiological changes in nutrient uptake induced by soil heterogeneity (Hodge, 2004) may also have contributed to our results, but we did not assess this.

Our second hypothesis – that elevated CO_2, soil nutrient heterogeneity and overall nutrient availability, alone and in combination, will promote changes in the composition of assemblages – was partially supported by our results. Overall, CO_2 and nutrient heterogeneity did not singly influence the species biomass responses. However, significant or marginally significant $CO_2 \times$ nutrient heterogeneity interactions were found for all species except *A. odoratum*. When they occurred, the direction of these interactions was species-specific (Fig. 4). Species-dependent differences in the response of aboveground biomass to elevated CO_2 were also evident when evaluating the results of the WinRatio index (Fig. 5). While the dominant species, *H. lanatus*, was the species that responded most to elevated CO_2 under homogeneous conditions at the medium and highest nutrient availability conditions, under heterogeneous conditions the subordinate species were those that responded most to elevated CO_2. In a review of competition experiments, Poorter & Navas (2003) found that C_3 grasses and N_2-fixing species were the groups of species that benefited most from elevated CO_2 (values of WinRatio index significantly >1) under high- and low-nutrient conditions, respectively. Interestingly, and contrary to this general pattern, we found that the N_2-fixer *T. repens* was a 'winner' under high nutrient availability conditions, but only when the nutrients were supplied heterogeneously. Previous studies have found that *T. repens* responds to both increases in soil nutrient availability and heterogeneity by increasing biomass growth (Turkington *et al.*, 1991; Hutchings *et al.*, 1997), but the joint effects of both variables on this species when growing in competition have not been evaluated before. Our results are consistent with these studies, and suggest that a plastic response of *T. repens* to the joint presence of increased nutrient availability and heterogeneity may be nonadditive under elevated CO_2. As legumes can transfer the N fixed from the atmosphere to other species, they are of paramount importance as drivers of community-wide responses to elevated CO_2 (Navas *et al.*, 1997; Warwick *et al.*, 1998; Stöcklin & Körner, 1999). Thus the evaluation of the role of soil nutrient heterogeneity as a driver of legume responses to joint increases in the atmospheric concentration of CO_2 and nutrient availability merits further study.

Consistent with the findings of Berntson *et al.* (1998) for a model, annual grassland community, the enhancement of N uptake was linearly related to biomass production. This suggests that the ability of plants to increase N uptake may be an important determinant of which species in an assemblage will be able to respond to increased CO_2 levels with increased biomass production. Notably, the greatest enhancement of both biomass and nutrient uptake under elevated CO_2 was found for the subordinate species under conditions of high nutrient availability and heterogeneous nutrient supply. Others (Navas *et al.*, 1997; Berntson *et al.*, 1998; Stöcklin & Körner, 1999) have also found that subordinate species are the most responsive to an increase in the concentration of atmospheric CO_2. Our results add to this literature by emphasizing that such responses may be dependent on both soil nutrient heterogeneity and availability. If the patterns observed in our experiment hold true, species composition is likely to change under elevated CO_2 conditions, in a manner that is dependent on nutrient availability and spatial pattern. Changes in species composition under elevated CO_2 have been detected in both field and microcosm studies (Berntson *et al.*, 1998; Joel *et al.*, 2001; He *et al.*, 2002), but none of these studies has explicitly considered soil nutrient heterogeneity as a factor in the outcome of these changes.

In the field, heterogeneity in resource distribution arises as a result of organic inputs (derived from leaf litter, dead roots, animal corpses, etc.) and their subsequent decomposition. Given that we used natural soil and organic material, the decomposition of the organic material in our experiment may resemble that observed in the field (Hodge, 2004). However, our approach is not without limitations. For example: (a) growing assemblages in PVC piping may alter root foraging responses because of physical restriction of lateral root growth (Fransen *et al.*, 1999); (b) patterns of patch heterogeneity and degree of contrast to the background soil may not reflect those found in the field; and (c) standardized climatic conditions (light, temperature, humidity, water supply) may amplify plant responses to soil nutrient availability and heterogeneity over those that may be observed in the field. The latter limitation is applicable to many controlled-environment studies and, when taken together with the first two limitations, it is clear that extrapolation of our results to the natural world should be done with caution. What we demonstrate are

potential plant responses to simultaneous changes in elevated CO_2, nutrient heterogeneity and nutrient availability (Jones *et al.*, 2000).

Given the multifactorial nature of global change (Houghton *et al.*, 2001), there is a need to include additional environmental factors in experiments devoted to understanding or predicting the biological effects of CO_2 enrichment (Körner, 2001). Despite the large number of studies conducted to assess the ecological consequences of soil nutrient heterogeneity, it has been an overlooked experimental factor in elevated CO_2 research. Recently, it has been emphasized that the interpretation of plant responses to elevated CO_2 should exclusively be in the context of the nutrient availability experienced by the species or assemblages under study (Körner, 2003). We strongly support this recommendation, and suggest that soil nutrient heterogeneity may also be of paramount importance in determining plant responses to elevated CO_2. Future field studies should consider explicitly how nutrient heterogeneity may influence the responses of plant species and assemblages to elevated CO_2 and nutrient availability. To achieve this, two different approaches may prove useful: (a) experimental addition of nutrient patches to field soils; and/or (b) consideration of the intrinsic nutrient heterogeneity within experimental plots as a covariate. Such studies will undoubtedly increase our ability to predict how species and ecosystems will respond to ongoing increases in atmospheric CO_2 concentration and nutrient availability in the real, and highly heterogeneous, world.

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Supplementary material

The following supplementary material is available for this article online.

Fig. S1 Above-ground biomass for the different species forming the assemblages. Data represent means and SE (n = 6). Note the differences in the y-axis between species.

Table S1 Summary results of the four-way nested ANOVA for assemblage-level measurements.

Table S2 Summary results of the four-way nested ANOVA to test for the effects of the different treatments on the proportion of the total aboveground biomass contributed by each species.

Table S3 Summary results of the four-way nested ANOVA to test for the effects of the different treatments on the foliar nitrogen concentration of each species.

Table S4 Summary results of the four-way nested ANOVA to test for the effects of the different treatments on the foliar C : N ratio of each species.

Table S5 Summary results of the four-way nested ANOVA to test for the effects of the different treatments on the proportion of nitrogen captured from the organic matter added by each species.

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