Response of *Fagus sylvatica* and *Picea abies* to the interactive effect of neighbor identity and enhanced CO_2 levels

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Key Message

Enhanced levels of CO_2 affected both the nutrition and morphology of both species. The effect of interspecific competition was dependent on the species identity but not on the CO_2 level.

Abstract

The interest in adaptive forest strategies to overcome predicted scenarios of climate change is increasing worldwide. An example of these strategies is the introduction of native species into mono-specific plantations. However, to fully consider this option/strategy, a higher understanding of the responses of forest tree species to concurrent biotic and abiotic factors is needed. The aim of the present study was to assess nutritional and morphological adjustments of individuals of European beech (*Fagus sylvatica* L.) and Norway Spruce (*Picea abies* (L.) Karst) growing at enhanced levels of CO_2 and with different proportions of con-specific individuals in its vicinity. Individuals that grew at elevated CO_2 levels showed higher values of relative growth rate (RGR), total twig dry biomass and root biomass, and lower values of leaf area ratio, leaf N and Mg concentrations and soil nutrient concentrations. Individuals of Norway spruce growing in the vicinity of high proportions of European beech showed a reduction in the allocation of biomass to foliar tissue and lower values of RGR and root biomass. European beech, by contrast, showed a limited response to Norway spruce presence and higher capacity in the exploitation of space both above- and below-ground. In conclusion, the lower response of European beech to both environmental factors suggests that the introduction of European beech into Norway spruce stands could be a feasible option in current forest transition strategies.

Keywords: Mixed forest, climate change, root morphology, growth, non-structural carbohydrates, CO_2 fumigation, plant-to-plant interactions.

1. Introduction

Introducing autochthonous species into mono-specific plantations is a successful way to increase diversity, reduce natural damages and attenuate the risk of investments under predicted climatic scenarios (Zerbe 2002; Gärtner and Reif 2004; Knoke et al 2005; Seidl et al 2009). The awareness of developing adaptive forest management strategies is mainstream in Europe and worldwide (Bolte et al 2009). However, contrasting results exist regarding whether mixed plantations can achieve greater productivity than monocultures (Pretzsch and Schütze 2009; Pretzsch et al 2010). And the consideration of a lower profitability of mixed-forest than monocultures is widespread among stakeholders and forest economists (Knoke et al 2008). In this context, a better understanding of the responses of forest tree species to interspecific interactions in mixed-forest is necessary to predict their long term sustainability and test if mixed-forest can be a sustainable management option (Pretzsch et al 2014).

The need to develop adaptive strategies is especially relevant for commercial mono-specific forest plantations of coniferous in Europe, where a severe reduction of forest stand's resistance against storm, snow, ice, drought and insect damage has been observed (Spiecker 2003). For example, mono-specific plantations of Norway spruce (*Picea abies* (L.) Karst), one of the most commercially valuable tree species in Europe, are declining (Jonard et al 2012; Bošel'a et al 2014). However, the survival of Norway spruce can be increased when growing in mixed-forest (Griess et al 2012). The introduction of European beech (*Fagus sylvatica* L.) into coniferous mono-specific stands plays a central role in current forest transition strategies (Geßler et al 2007). European beech is one of the most abundant and dominating tree species of the potential natural vegetation in temperate forests of Europe and several methods exist for the conversion of pure Norway spruce stands into mixed stands with European beech (Bravo-Oviedo et al. 2014). However, the long term sustainability of mixed stands depends on factors such as species identity or site conditions that can define the net outcome of mixing different species (Pretzsch et al 2010). In addition, available information on how different species in mixed-forest responds to concurrent biotic interaction under predicted scenarios of climate change, such as the increase of atmospheric CO_2, is much more limited (Geßler et al 2007; IPCC 2007; Smith et al 2013b).

Under elevated CO_2 conditions, tree species can increase plant growth directly by enhancing photosynthetic capacity or indirectly by increasing water or nutrient use efficiency (Körner 2006). However, the fertilization effect of CO_2 may be limited by the progressive scarcity of nutrient supplies, especially N (Reich et al 2006; Dieleman et al 2010) or by a lack of positive growth response despite the stimulation of the C assimilation rate induced by CO_2 (Körner 2006; Kirschbaum 2011). Additionally, in natural conditions, the response of an individual can depend on the identity of neighboring species (Lau et al 2010) because processes affecting growth, such as nutrient acquisition, can be contingent on the identity of neighboring species under enhanced levels of CO_2 (Friend et al 2000; Zak et al 2012). These idiosyncratic responses to high CO_2 levels question the reasonable expectation that only superior competitors would be benefited in a mixture of tree species. Assessing how interspecific competition and high levels of CO_2 affects individual tree performance can help to understand the response of mixed-forest under future climate scenarios (Niinemets 2010).

The net outcome of interspecific interactions between broadleaved and coniferous species, specifically between European beech and Norway spruce, under enhanced CO_2 levels is still largely unclear. At the sapling stage, Norway spruce can benefit from high CO_2 levels and outcompete European beech (Kozovits et al 2005). But, the combined effect of CO_2 levels and other environmental factors, especially soil resources, can also mediate interspecific relations leading, for instance, to European beech to benefit from high CO_2 levels and outcompete Norway spruce under certain circumstances (Spinnler et al 2002). Early differences in growth or morphology between both species can determine their performance at the adult stage where differences in crown structure or in the efficiency to occupy space per structural cost can likely affect the resilience of the stand to disturbances (Reiter et al 2005; Pretzsch 2014). Nevertheless, our understanding of how interspecific relations vary as the proportion of neighbors of a different species identity change is still scarce. Most studies analysing interspecific interactions between European beech and Norway spruce are based on 1:1 mixtures. This ratio seldom occurs outside controlled conditions. Thus, the application of knowledge acquired in 1:1 mixtures to natural conditions, where different mixture ratios are common, is limited. Testing different mixtures of European beech and Norway spruce may provide more comprehensive knowledge on the underlying mechanism that operates between both species and facilitate the generalization of the results of experiments under controlled conditions to more natural and realistic situations.

The aim of the present study was to assess how interacting relations of European beech and Norway spruce respond to CO_2 environment and if this effect varies when the proportion of neighboring individuals of a different identity change. We evaluated growth, biomass allocation to foliar or supporting tissue at the twig level, belowground morphological parameters and nutritional conditions in saplings of European beech and Norway spruce exposed to six years of CO_2 enrichment growing at different admixture treatments. In addition, we assessed soil nutrient concentrations to evaluate if the responses of the individuals to CO_2 environment and admixture treatments were modulated by this resource. Specifically, we aimed to answer the following questions: i) Is the individual response to CO_2 enrichment dependent on the proportion of neighboring species of a different identity growing in its vicinity or ii) are both pressures acting independently? iii) Are the observed responses contingent on the species identity? iv) Is there any mediating effect of soil nutrient concentration? We hypothesized that individuals of different species identity can respond differently to CO_2 environment, but this effect may vary between different levels of admixture. Studying the response of individual plants to intra and interspecific mixtures can be of key interest to better planning the management of mixed-forest where different levels of admixture occur.

2. Material and Methods

2.1. Study design and sampling scheme

The experiment was carried out in Beskydy Mountains (Bílý Kříž; 49°30′N, 18°32′E, 908 m a.s.l., NE of the Czech Republic). In this region, planted Norway spruce forests are the predominating vegetation type. The altitudinal range varies between 500 and 1700 m above sea level (a.s.l). A moderately cold and wet mountain climate is typical for this region, annual average temperature of the study site is 4.9 ºC and yearly accumulated rainfall is 1100 mm. Soils of the study site are characterized as ferric podzols. Soil depth is moderate and clay fraction range between 15-35 %. The geological bedrock is formed by Mesozoic Godula sandstone (flysch type).

The measurements were carried out in two adjoined glass domes (10 x 10 m in length and 7 m height in the central part), at ambient (385 μmol CO_2 mol^{-1}) and elevated (700 μmol CO_2 mol^{-1}) CO_2 concentrations. Glass domes resembled the well-known open-top chambers (OTCs) but that included a few modifications; such as adjustable windows (72 in total) made of safety glass that can be opened in 10º steps from fully close to fully open. The modifications were designed to overcome the problems of strong wind, humidity and temperature gradients associated to OTCs. On average, temperature and humidity gradients were maintained inside the domes at similar levels than natural conditions for more than 80 % of the time. The target elevated CO_2 level was maintained within the range of 600 - 800 μmol CO_2 mol^{-1} *ca.* 72 % of the time of the growing season. Soils inside the glass domes were not mechanically modified and are natural soils from the study zone. The proximity between both glass domes (less than 2m) makes unlikely *a priori* differences in soil properties between them. Further description of the chambers design and installation can be found in Urban et al, (2001).

A total of 99 seedlings per chamber were planted in 2006 in eleven lines with a separation of 1 m between plants and 0.85 m between lines. On average the stem diameter at the time of planting of Norway spruce and European beech seedlings was 10.1 ± 0.26 mm and 11.3 ± 0.45 mm (mean ± S.E) respectively. Plant species were distributed to achieve the different admixtures treatments. There were three admixture treatments that were defined according to the number of neighbors belonging to the same species identity as the individual that was sampled (hereafter focal) (Figure 1). Briefly, mono-specific (MS): all neighbors are individuals of the same species; group-mixture (GM): half of the neighbors are con-specific and the other half not; single-

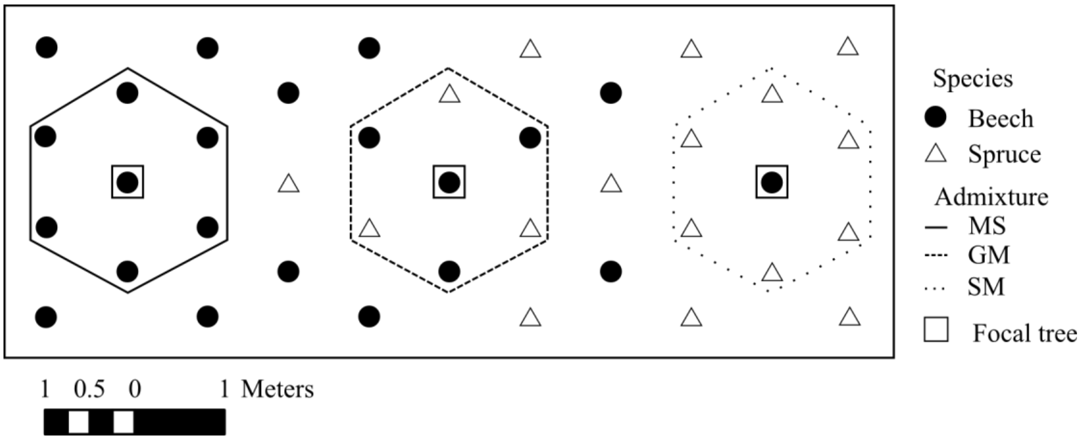

Figure 1 Schematic representation of admixture treatment layout, mono-specific (MS), group-mixture (GM) and single-mixture (SM), per CO_2 treatment showing European beech as a focal tree and Norway spruce as neighbor species. The distribution of Norway spruce was the same but it is not shown for simplicity.

mixture (SM): no neighbors from the same specie (Figure 1). In summary, a total of 48 individuals were sampled (2 chambers x 2 species x 3 admixture treatments x 4 individuals). All sampled plants were surrounded by six individuals and no focal plants were located in the first lines close to the walls to avoid edge effects.

2.2. Plant growth and components of biomass allocation at the twig level

Sapling stem diameter was measured yearly from 2006 to 2012 in each individual at the end of the growing season. Stem diameter was always measured in the same zone (30 cm height) by marking a line around the sapling stem. In 2012 once primary growth was fully achieved and during two consecutive days, current-year apical twigs were cut from four branches, one per orientation, to assess variation of aboveground biomass allocation among species and treatments. Branches were selected approximately at the same sapling height (1.3 m), corresponding to the upper third of the canopy in all cases. Twigs clearly located underneath other branches were not sampled to avoid confounding effects due to shadow.

From each twig, leaf/needle was removed and scanned (Epson Expression 10000XL) and projected leaf/needle area (LAp cm^2) was measured with ImageJ (1.46r). All plant material was oven-dried (65 ºC for 48 h) and weighed. Twig leaf/needle area ratio (LAR cm^2 g^{-1}) was determined by dividing LAp by the total twig dry weight (TW g), including all leaf/needle and twig. Leaf/needle mass fraction (LMF %) was calculated by dividing total leaf/needle dry weight by total twig dry weight.

2.3. Fine root sampling and processing

Soil samples were collected from the uppermost soil layer (15 cm) by means of a soil core (bipartite root auger Eijkelkamp, NL) in 2012, on the same days as twig sampling. One sampling point was placed randomly around each focal tree approximately half way between the stem and the drip line of each individual, but no closer than 20 cm to the stem. Soil samples were kept fresh at 4 ºC until analysis. Root samples were separated from soil and washed using different filters (mesh size between 2 and 0.125 mm) in order to avoid fine roots losses. Soil was stored and dried for subsequent chemical analysis (see below). All roots less than or equal to 2 mm in diameter were classified as fine roots. Coarse roots and roots with symptoms of being dead (dark discoloration of the central cylinder and a decreased flexibility of root segments) were excluded from the analysis. Fine roots were identified visually and separated manually per species according to Schmid (2002). Briefly, *Picea* roots are elastic with a relatively thick and irregularly structured brownish cortex, whereas *Fagus* roots are less elastic and the red-brown cortex is thin with lines along the longitudinal axis. Root material was scanned (Epson Expression 10000XL) and images were processed with WinRhizo^{TM} (Régents Instruments Inc., Canada) to assess root morphological attributes: root length density (RLD cm / dm^3) and average diameter (AD, mm). Subsequently, roots were oven-dried (65 ºC for 48 h) and weighed. Differences in the abundance of both species inherent in our study design could preclude the direct comparison of fine root attributes between admixture treatments. Although focal trees were sampled under its canopy, a higher proportion of the opposite species identity in its vicinity may increase the probability of finding its roots in the sample. To avoid this bias, the standardization of total core values of Norway spruce and European beech to one unit of total basal area for the corresponding species surrounding the sample point has been showed to be an effective adjustment (Schmid 2002; Bolte and Villanueva 2006).

2.4. Chemical analysis

Composite leaf/needle samples from the four twigs per individual were ground to pass a 250 µm mesh. Total nitrogen (N) and carbon (C) were measured by high-temperature (1000 °C) dry combustion method with an automatic analyser LECO CNS 2000. Nutrient concentrations (P, Mg, Ca and K) were measured with an ICP analyser (ICP-OES, Yobin Yvon Última 2, Tokyo, Japan) after wet-digestion with H_2SO_4/H_2O_2 (Temminghoff and Houba 2004). Soil samples were sieved to pass a 2 mm mesh. Fine-earth was used to analyse total nitrogen and carbon with the same method used for leaves/needles, UV-visible spectrophotometry with Mehlich 3 extraction was used for P determination, and the Ca, Mg and K were determined by atomic absorption spectrophotometry with ammonium acetate extraction.

Leaf/needle and fine root non-structural carbohydrates concentrations (NSC) were determined colorimetrically by the anthrone method (Fales 1951) using glucose as standard. Ethanol-soluble fraction was extracted with 80% ethanol at 30 ºC during 30 min and the residue was boiled for 3 h at 100 ºC with 3% HCl to hydrolyze starch. Final values were found as the sum of ethanol-soluble carbohydrates and starch. NSC analyses were carried out in duplicate for each sample, further details on analyses and calculations can be found in Poorter and Villar (1997). Leaf NSC concentrations are contingent on the photosynthetic capacity of the leaves as well as the amount of irradiance that received (Lambers et al 2008). This, together with the time delay that exists between photosynthate production and translocation, may affect the content of leaf substances diurnally (Poorter et al 2009). To partial out this source of variability, it has been recommended to express leaf/needle nutrient concentrations on a NSC-free dry weight (Poorter and Villar 1997; Niinemets 1997). Therefore, we expressed leaf/needle nutrient content on a NSC-free dry weight basis as: Nut_c = Nut / (1 – NSC); where Nut is the original nutrient concentration and NSC is expressed as the proportion of dry matter.

2.5. Data analysis

To calculate sapling relative growth rate (RGR) we followed the recommendations of Paine et al (2012). For each individual we regressed basal diameter as a function of time and fit a power-law function to the data. Depending on the value of the exponent, power-law models satisfactorily allow RGR to decrease as biomass increases, a fundamental property for growth analysis. We calculated RGR values per sapling and year from the solution of the power-law differential equation as a function of time (Paine et al 2012). To analyse the effect of CO_2 treatment (A and E), species identity (European beech and Norway spruce) and admixture treatment (MS, GM and SM) on the temporal dynamics of RGR, we employed linear mixed models. To take into account the temporal autocorrelation of the dependent variable we included a temporal correlation structure in the model (Zuur et al 2009) and sapling as a random effect to avoid pseudo-replication. The final set of explanatory factors and the best temporal correlation structure (AR-1) were selected according to the lowest AIC.

To test the effect of the independent variables (CO_2, species identity and admixture) and their interactions on multivariate data sets of leaf morphology (twig weight, leaf area ratio and leaf mass fraction), leaf nutrient concentration (N, P, K, Ca and Mg), root system (root biomass, root diameter and root length density) and soil nutrient content (N, P, K, Ca and Mg) we performed permutational multivariate ANOVA using

Euclidean distance and 10000 permutations. Multivariate datasets were projected onto the first two Principal Component axes to assess visually the significant differences found between treatments in the permutational multivariate ANOVA. Subsequently, we tested the effect of the independent variables on each univariate response variable by means of linear mixed models, including sapling as random factor. To account for differences in sapling size in the effect of twig morphological and root system variables, we included stem diameter at the time of sampling as a covariate in the models. The consideration of sapling stem diameter as a proxy of size allowed us to avoid its confounding effect when testing main factor effects. When a significant interaction effect was found, Tukey-HSD post-hoc analyses were carried out. To explore the relationship per CO_2 treatment between overall soil nutrient content and sapling growth, Mantel tests were carried out. Dependent variables were transformed when necessary to comply with normality and homogeneity of variance assumptions. Results are expressed as mean values ± SE of the mean. All statistical analyses were performed in R v 3.0 (R Core Team 2013).

3. Results

3.1 Response of growth to admixture and CO_2 enrichment

Sapling growth showed independent responses to admixture and CO_2. Saplings showed higher values of RGR in the elevated environment of CO_2 than in ambient (1.21 ± 0.01 and 1.18 ± 0.01 cm cm^{-1} $year^{-1}$; F = 4.1 $P = 0.049$) throughout the study period (Fig. 2) and this effect was consistent among admixture treatments and species (no significant interaction between CO_2 and admixture or CO_2 and species). The effect of admixture on RGR was only apparent for Norway spruce (marginally significant interaction between species and admixture treatment, F = 2.9 $P = 0.062$). Indeed, the saplings of Norway

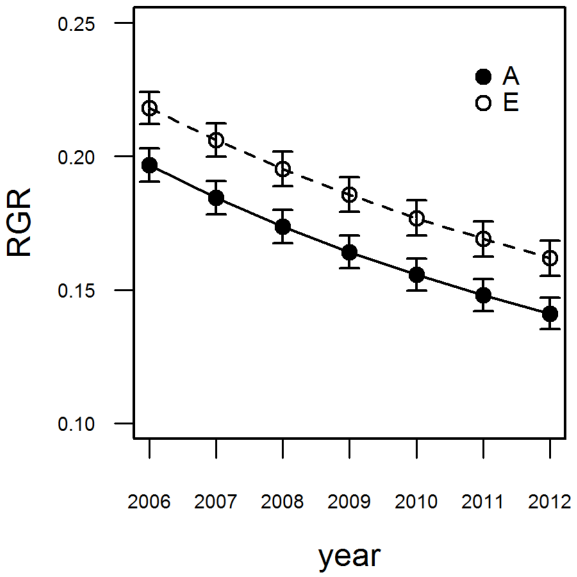

Figure 2 Temporal dynamic of RGR based on stem diameter of European beech and Norway spruce growing at elevated (E) or ambient (A) CO_2 treatments.

spruce showed lower RGR values when growing in SM than in MS (1.17± 0.01 and 1.24 ± 0.01 cm cm^{-1} $year^{-1}$, *P* = 0.063), while RGR values of European beech showed no response to admixture (1.18 ± 0.01 and 1.17 ± 0.01 cm cm^{-1} $year^{-1}$, *P* = 0.989).

The relationship between soil nutrient concentration and RGR was dependent on the CO_2 treatment. At ambient levels of CO_2, we observed a positive relationship between soil nutrient concentration and RGR (Mantel r = 0.13 *P* = 0.071), yet RGR was negatively related with soil nutrient concentration in the elevated CO_2 environment (Mantel r = -0.21 *P* = 0.004).

3.2 Multivariate response of European beech and Norway spruce to CO_2 enrichment and admixture

The effect of CO_2 treatment explained a high proportion of the multivariate variation across variable groups (Table 1). Soil nutrient concentration and root system attributes were significantly affected by CO_2 treatment but their responses were dependent on species identity and species identity and admixture treatment, respectively. Leaf/needle nutrient concentration and twig morphology showed a lesser response to CO_2 environment, with admixture treatment explaining a higher portion of the variance, but the effect of admixture seemed to depend on the species identity of the focal sapling (Table 1). The projection of twig morphology and leaf/needle nutrient concentration on a reduced space suggested that Norway spruce was more affected by admixture than European beech (Fig. 3). Indeed, we observed larger differences in the morphology of Norway spruce twigs and leaf/needle nutrient concentrations between MS and SM admixture treatments (larger separation between centroids) than in European beech (Fig. 3).

Figure 3 Projected values of leaf morphology (A) and leaf nutrient concentration (B) multivariate dataset onto the first two principal component showing the interacting term between species (European beech and Norway spruce) and admixture , mono-specific (MS), group mixture (GM) and single mixture (SM). The explained variance per axis is shown. Open symbols represent each sapling per admixture treatment. Filled symbols represent the centroid per admixture treatment connected by lines.

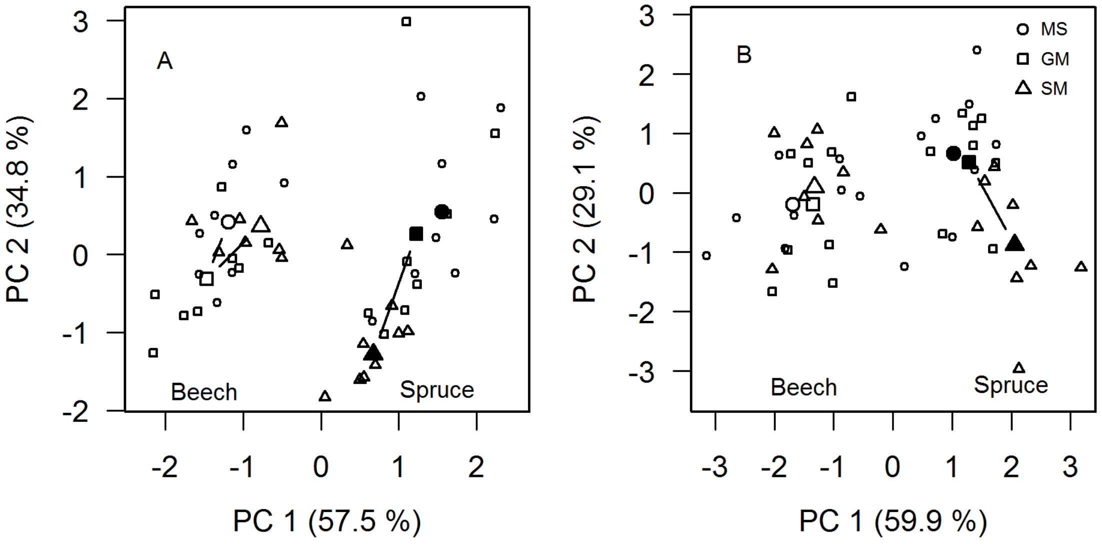

Table 1 Results from perMANOVA testing the effect of CO_2 (E), Species (Sp), Admixture (Ad) and their interactions on multivariate datasets of leaf morphology (twig weight, leaf area ratio and leaf mass fraction), leaf nutrient concentration (N, P, K, Ca and Mg), root system attributes (root biomass, root diameter and root length density) and soil nutrient content (N, P, K, Ca and Mg). Significant differences (P < 0.05) are depicted in bold font and marginal significant in bold and italic (0.05 < *P* < 0.10).

		CO_2 (E)	Species (Sp)	Admixture (Ad)	E:Sp	E:Ad	Sp:Ad	E:Sp:Ad	Residual
Leaf morphology	R^2	0.008	0.863	0.009	0.001	0.005	0.036	0.0	0.074
	F	4.0	419.0	2.3	0.7	1.3	8.7	0.2	
	P	***0.054***	**<0.001**	0.106	0.401	0.270	**<0.001**	0.786	
Leaf Nutrients	R^2	0.023	0.455	0.080	0.002	0.023	0.099	0.027	0.287
	F	2.9	56.0	5.0	0.2	1.4	6.2	1.6	
	P	***0.067***	**<0.001**	**0.003**	0.747	0.216	**0.001**	0.173	
Root system	R^2	0.067	0.033	0.321	0.004	0.007	0.032	0.099	0.433
	F	5.6	2.7	13.0	0.3	0.3	1.3	4.1	
	P	**0.022**	**0.099**	**<0.001**	0.539	0.742	0.271	**0.020**	
Soil Nutrients	R^2	0.307	0.053	0.021	0.057	0.028	0.017	0.022	0.491
	F	22.	3.9	0.7	4.2	1.0	0.6	0.8	
	P	**<0.001**	***0.052***	0.470	**0.047**	0.368	0.542	0.457	

3.3 Response of European beech and Norway spruce to CO_2 enrichment

Further analysis of each univariate variable separately confirmed the effects of high CO_2 levels observed across multivariate datasets (Table S1). Saplings growing in the elevated CO_2 environment had higher values of root biomass (3.25 ± 0.66 and 2.17 ± 0.54 g m^{-2} cm^{-2} BA; F = 4.9 $P = 0.031$), total twig biomass (1.06 ± 0.10 and 0.85 ± 0.06 g; F = 4.5, $P = 0.039$) and leaf/needle P concentration than in ambient (Table 2). Root length density values of European beech also showed a significant response to CO_2 environment, showing higher values in the elevated environment than in ambient (0.76 ± 0.17 and 0.32 ± 0.05 cm dm^{-3} cm^{-2} BA; F = 3.4, $P = 0.003$). On the other hand, we observed the opposite response to CO_2 levels, with significant lower values in the elevated environment than in ambient, for leaf/needle area ratio (108.72 ± 12.14 and 119.01 ± 11.4 cm^2 g^{-1}; F = 4.3, $P = 0.045$), soil nutrient concentration, that was consistently lower for most of the nutrients analysed (Figure 4), and leaf/needle N and Mg concentration (Table 2).

Higher levels of CO_2 did not affect either leaf/needle C or NSC concentration. Norway spruce saplings showed higher root NSC concentrations in the elevated environment than in ambient, whereas European beech did not show any response (significant interaction between CO_2 treatment and species, F = 5.7 $P = 0.002$) (Figure 5). The response of leaf/needle nutrient stoichiometric to CO_2 environment was also significant, showing lower values of C:P and N:P ratios at high levels of CO_2 than in ambient (Table 2).

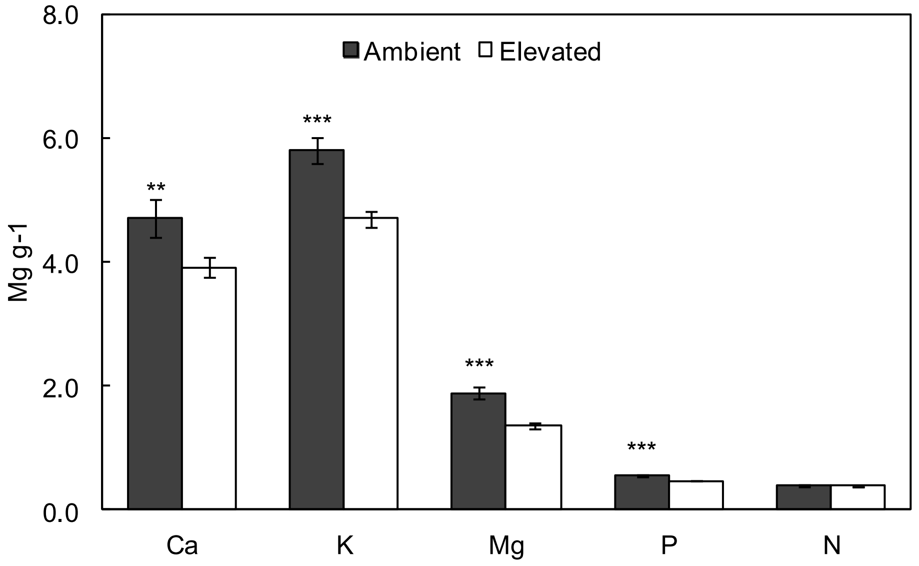

Figure 4 Soil nutrient concentration (mg / g) mean values (± S.E) per CO_2 treatment, ambient and elevated. Asterisks depict significant differences at: *0.05< P < 0.1, ** P < 0.05 and *** P < 0.01.

Table 2 Needles and leaves nutrient concentration (mg/g) (± S.E) per CO_2 treatment. Leaf/needle nutrient concentrations are corrected values taking into account NSC content (further details in the text). Significant values are depicted in bold ($P < 0.05$) and marginally significant in bold and italics ($0.05 < P < 0.1$).

	Leaves / Needles			
	Ambient	Elevated	F	P
N	19.30 ± 0.33	18.36 ± 0.43	2.8	***0.098***
P	2.09 ± 0.12	2.31 ± 0.09	3.9	***0.051***
K	8.78 ± 0.64	8.2 ± 0.65	2.2	0.143
Mg	1.84 ± 0.10	1.7 ± 0.10	3.2	***0.082***
Ca	7.65 ± 0.40	7.73 ± 0.56	0.1	0.892
C (%)	50.52 ± 2.33	50.17 ± 2.34	2.0	0.165
C:N	26.37 ± 0.51	27.96 ± 0.69	2.3	0.136
C:P	261.85 ± 16.4	228.08 ± 9.41	6.1	**0.018**
N:P	10.03 ± 0.69	8.18 ± 0.33	12.1	**0.001**

Table 3 Values (mean ± S.E) of twig morphological, total twig weight (g), leaf mass fraction (%), leaf area ratio (cm^2 / g), and root system, average diameter (mm), biomass (g / m^2 cm^2 BA), length density (cm / dm^3 cm^2 BA), attributes of European beech and Norway spruce, growing at different regimes of admixture, mono-specific (MS), group-mixture (GM) and single-mixture (SM). Different letters per species and row indicated significant differences at $P < 0.05$.

	Species	Admixture			Interaction	
Twig Morphology		MS	GM	SM	F	*P*
Total dry weight	Beech	1.24 ± 0.10	0.96 ± 0.1	1.14 ± 0.1	3.4	0.043
	Spruce	1.03 ± 0.14a	0.97 ± 0.19a	0.40 ± 0.05b		
Leaf mass fraction	Beech	0.72 ± 0.01	0.75 ± 0.02	0.69 ± 0.02	5.2	0.009
	Spruce	0.59 ± 0.02b	0.62 ± 0.02b	0.68 ± 0.01a		
Leaf area ratio	Beech	165.2 ± 7.9ab	178.68 ± 9.2b	155.9 ± 6.0a	6.5	0.004
	Spruce	46.66 ± 3.21b	55.92 ± 4.32b	80.82 ± 6.14a		
Root System						
Average diameter	Beech	0.29 ± 0.03	0.28 ± 0.01	0.28 ± 0.02	4.6	0.015
	Spruce	0.44 ± 0.03a	0.38 ± 0.03a	0.29 ± 0.02b		
Root length density	Beech	0.86 ± 0.23a	0.37 ± 0.08b	0.39 ± 0.13b	3.8	0.006
	Spruce	0.33 ± 0.05	0.26 ± 0.06	0.28 ± 0.06		
Root biomass	Beech	4.66 ± 0.81a	1.95 ± 0.46b	2.61 ± 0.65ab	5.9	<0.001
	Spruce	4.13 ± 0.84a	2.02 ± 0.48b	0.89 ± 0.23b		

3.4 Response of European beech and Norway spruce to admixture

The effect of admixture was mostly limited to twig morphological and root system attributes. In general, Norway spruce showed a higher response to the presence of European beech than European beech to the presence of Norway spruce (Table 3). At the twig level, Norway spruce saplings showed significant lower values of total twig biomass and significant higher values of needle mass fraction and needle area ratio in SM than in MS. On the contrary European beech showed significant lower values of leaf area ratio in SM than in MS (Table 3). Both species showed significant higher values of root biomass in MS than in SM. Norway spruce showed a significant reduction of root average diameter and no effect on root length density between MS and SM. By contrast, European beech showed a significant reduction in root length density and no effect on average diameter between both treatments.

4. Discussion

4.1. Response of plant growth and twig morphological attributes to CO_2 and admixture

Enhanced levels of CO_2 and interspecific competition modified the allocation of resources in European beech and Norway spruce; however the amount of change depended either on the CO_2 environment or on the admixture treatment. According to our results, the response of each species type to admixture would be similar at both levels of CO_2, enhanced and ambient. In other words, an individual of Norway spruce diminished its growth when growing in the vicinity of high proportions of European beech and this effect persisted under elevated CO_2 conditions. This result contradicted our expectations because Norway spruce has been commonly regarded as a superior competitor than European beech (Matyssek et al 2005) and examples of Norway spruce outcompeting European beech at elevated CO_2 levels have been reported (Kozovits et al

2005). The highest response of Norway spruce was observed when growing in high densities of European beech, suggesting that shading could have limited Norway spruce growth (Kirschbaum 2011). Acclimation of plants to shade involves higher allocation to leaf area per gram plant, among other adjustments. The higher values of leaf/needle area ratio or leaf/needle mass fraction in SM than in MS observed in Norway spruce, but not in European beech that showed the opposite pattern (Table 3), supports the notion of shading induced by European beech. The tendency to conserve resources in plants growing in shade, could explain the lower RGR values observed in Norway spruce than in European beech.

Results of European beech outcompeting Norway spruce have only been shown in nutrient rich calcareous soils (Spinnler et al 2002). Interestingly, the soils of our studied zone are mostly acidic (pH ~ 4) suggesting that other factors may play a major role in determining the direction of this interspecific interaction. These discrepancies may arise due to the different admixture treatments employed in both studies. The lack of differences observed in our study between MS and GM admixture treatments suggests that experiments assessing the effect of admixture at 1:1 proportions may not fully disentangle the effect of biotic interactions and be more dependent on external factors, such as soil type.

The adjustments of both tree species to admixture seemed to conflict with the overall effect of CO_2 enrichment on aboveground resources allocation, especially in Norway spruce. While admixture tended to optimize light harvesting by increasing the allocation of resources to leaves, CO_2 fumigation tended to induce the opposite. We observed that both species showed lower values of leaf area ratio when growing at enhanced levels of

CO_2. A proportionally higher reduction in the amount of photosynthetically active tissues and a higher sequestration into structural and conducting tissues may be related to processes of downward regulation (Poorter and Navas 2003). This result would be in accordance with the observed reduction in N and Mg concentration at high CO_2 levels and would point to a diminution of the photosynthetic capacity of the studied trees (Lukac et al 2010). Conflicting requirements for biomass investment in different plant organs to increase the tolerance to different stresses are common in nature and may lead to certain cost and risk that may compromise plant growth and survival (Valladares and Niinemets 2008). Processes of down-regulation are often attributed to imbalances between source-sink relationships (Urban et al 2003). However, we did not observe differences in the accumulation of NSC on leaf/needle between CO_2 treatments (Fig. 5), suggesting a weak downward regulation effect in the leaf/needle cohort studied (Tissue et al 2001) and limited consequences of the conflicting effect between environmental stresses.

4.2. Response of root system attributes to CO_2 and admixture

The increase in root biomass in both species at high levels of CO_2 suggests a shift in the allocation of carbon to the root system (Norby *et al.* 2002). This flux could be especially relevant in Norway spruce which showed a significant increase in the concentration of NSC in roots. Indeed, we observed a significant correlation between root biomass and NSC concentration for Norway spruce ($r = 0.71$, $P = 0.009$), whereas we did not observe this effect in European beech ($r = 0.52$, $P = 0.175$). High root NSC concentrations in trees exposed to high levels of CO_2 can be interpreted as an accumulation of substrate available to support the production of fine-roots (Norby et al. 2002). However, both species increased their fine root biomass at elevated levels of CO_2

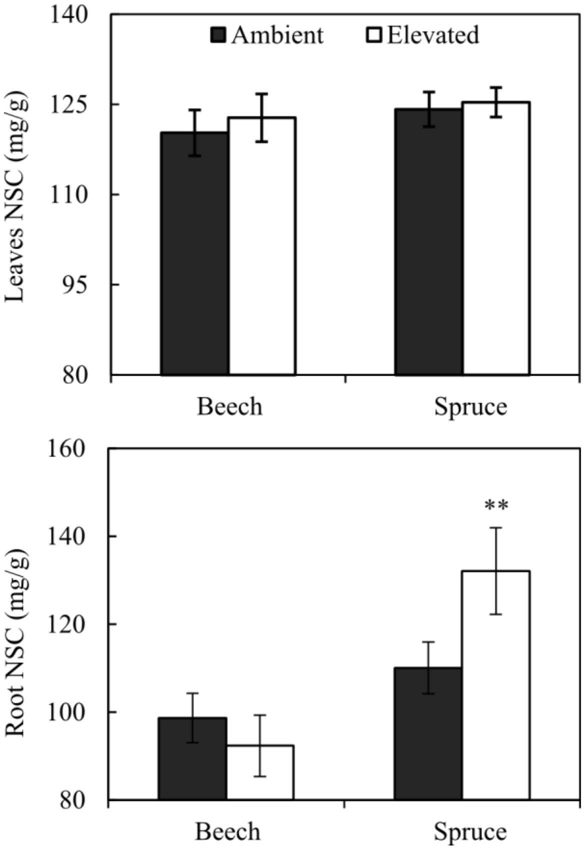

Figure 5 Leaves / needles and roots non-structural carbohydrates concentration (NSC, mg / g) mean values (± S.E) of European beech and Norway spruce per CO_2 treatments, ambient and elevated. Asterisks depict significant differences at $P < 0.05$.

similarly, suggesting that these discrepancies could be related to a different strategy of biomass allocation to roots between species. Similar to the aboveground compartment, European beech tended to use a higher volume of soil, especially at elevated levels of CO_2. Indeed, European beech accompanied the increase in biomass by an increase in root length density, suggesting a higher use of belowground space under competitive environments (Grams et al 2002; Bolte and Villanueva 2006). The effect of admixture on root attributes also support the notion of a different belowground strategy between both species. This effect together with the discrepancies observed at the twig level, could be an additional evidence of the negative consequences of admixing Norway spruce trees within a group of European beech at early developmental stages (Bolte et al 2014).

4.3. Effect of CO_2 and admixture on nutrient relations

High levels of CO_2 tended to modify the nutrient relations of both species. The consistent lower values of soil nutrient concentration in the elevated CO_2 environment, together with its negative association with RGR, suggest an increase difficulty in the access to soil resources (e.g. by a diminution in nutrient availability) which may have elicited an increased soil exploration by fine roots (Norby and Iversen 2006; Smith et al 2013a). Extensive root systems exploring large volumes of soils are associated with the plant ability to capture less mobile elements, such as P, but not necessarily with more mobile nutrients, such as N (Sands and Mulligan 1990). In addition, the allocation of resources to the root system is not necessarily associated with a benefited nutrition and it might exacerbate the limitation of nutrients by enhancing the sink effect of other compartments, such as ectomycorrhizas and mycelia (Cudlin et al 2007). As a result, the observed higher allocation of resources to the belowground compartment did not guarantee a positive effect in leaf/needle nutrient concentrations, with the exception of phosphorus (Table 2). The positive effect observed in P nutrition may be related with a higher exudation of phosphatases which are related with P uptake and have been shown to be favored under CO_2 elevated environments (Duval et al 2012). Nutrient concentrations values are related to the quantity of nutrients still embedded in organic molecules or bound to the soil matrix, thus the significant lower values observed suggest that plants tried to increase their nutrient uptake not only by increasing the amount of explored soil but also by triggering additional mechanisms to scavenge nutrients (Phillips et al 2011). Nevertheless, despite the likely efforts to increase nutrient uptake, we observed a significant reduction in the N:P ratio in the elevated environment. Imbalances in nutrient stoichiometry of plant tissues are related to nutrient limitations and can have important implications for plant interactions with nutrient cycling (Elser et al. 2000; Güsewell 2004). Thus our results suggest that long-term fumigation may have an indirect effect on plant nutrition through the modification of litter quality, microbial community and the recycling of nutrients within the ecosystem.

5. Conclusions

The morphology and nutrition of European beech and Norway spruce saplings showed a significant response to elevated levels of CO_2. After six years of CO_2 enrichment both species showed higher relative growth rate at elevated than ambient CO_2 levels, but the lower values of soil nutrient concentrations together with the higher allocation of resources to the root systems suggest a likely nutrient limitation effect in the elevated environment. The effect of admixture was dependent on the species identity and mostly limited to morphological attributes. Our results suggest that experiments assessing the effects of species mixture at 1:1 ratios may not be as easily transfer to natural conditions as supposed because they can overlook certain species-specific adjustments that may arise only when each species is outcompeted. Overall, European beech seemed to cope better with both environmental pressures suggesting that the introduction of single individuals of this tree species into Norway spruce stands could be a feasible option in current forest transition strategies, at least at the sapling stage.

Author contributions statement

V.R. and E.A. designed the sampling scheme, collected and processed the samples and wrote the manuscript R.P. revised the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Supporting Information

S1 Results from best linear mixed models based on lowest AIC with leaf morphology variables (twig weight, leaf area ratio and leaf mass fraction), leaf nutrient concentration (N, P, K, Ca and Mg), root system attributes (root biomass, root diameter and root length density), non structural carbohydrates concentrations (NSC) and soil nutrient content (N, P, K, Ca and Mg) as dependent variables. All models included sapling as a random effect. Twig morphological and root system variables models included stem diameter as a covariate to account for the effect of sapling size. Dark grey cells depict significant effect at $P < 0.05$ and light grey cells marginally significant at $0.05 < P < 0.10$.

		CO$_2$ (E)	Species (Sp)	Admixture (Ad)	E:Sp	E:Ad	Sp:Ad	E:Sp:Ad
Twig	Twig biomass	■	■	■			■	
	Leaf Area ration	■	■	▒			■	
	Leaf Mass Fraction		■	■			■	
Root	Average Diameter		■	■			■	
	Root Length Density	■	■	■	■		■	
	Root Biomass	■		■			■	
Leaf/ needle nutrient conc.	N	▒						
	P	▒	■					
	K		■	■			■	
	Ca		■		■			
	Mg	▒	■					
	C							
	C:N							
	C:P	■	■		▒			
	N:P	■	■					
NSC	Leaf/needle							
	Root		■		▒			
Soil nutrient conc.	N							
	P	■						
	K	■						
	Ca	■						
	Mg	■	■		■			
	P	■						

Table S2 Values (mean ± S.E) of twig morphological, total twig weight (g), leaf mass fraction (%), leaf area ratio (cm^2 / g), root system, average diameter (mm), biomass (g / m^2 cm^2 BA), length density (cm / dm^3 cm^2 BA), leaf/needle nutrient concentration (mg/g), leaf/needle and root non structural carbohydrates concentrations (mg/g) and soil nutrient concentrations (mg/g) at Ambient and Elevated CO$_2$ levels of European beech and Norway spruce individuals. Marginally significant differences $0.05 < P < 0.1$ are depicted in italics.

		Ambient	Elevated	F	P-value
Twig	Twig biomass	0.28 ± 0.03	0.37 ± 0.04	4.3	0.037
	Leaf Area ration	119.01 ± 12.14	108.72 ± 11.41	4.2	0.046
	Leaf Mass Fraction	0.69 ± 0.02	0.66 ± 0.01	-	-
Root	Average Diameter	0.32 ± 0.02	0.33 ± 0.02	-	-
	Root Length Density	0.31 ± 0.03	0.52 ± 0.1	5.6	0.022
	Root Biomass	2.17 ± 0.38	3.25 ± 0.46	4.9	0.031
Leaf/ needle nutrient conc.	N	16.95 ± 0.29	16.11 ± 0.4	2.8	*0.098*
	P	1.83 ± 0.1	2.03 ± 0.08	3.9	*0.051*
	K	7.71 ± 0.56	7.19 ± 0.58	-	-
	Ca	6.72 ± 0.35	6.78 ± 0.49	-	-
	Mg	1.62 ± 0.09	1.49 ± 0.09	3.2	*0.082*
	C	495.45 ± 2.05	491.38 ± 2.06	-	-
	C:N	29.45 ± 0.56	31.0 ± 0.84	-	-
	C:P	0.0037 ± 0.0002	0.0041 ± 0.0002	6.0	0.017
	N:P	10.03 ± 0.69	8.18 ± 0.33	12.1	0.001
NSC	Leaf/needle	51.75 ± 1	52.58 ± 0.97	-	-
	Root	104.35 ± 4.14	112.19 ± 7.22	5.8	0.002
Soil nutrient conc.	N	0.41 ± 0.01	0.39 ± 0.01	-	-
	P	0.56 ± 0.01	0.48 ± 0.01	19.6	<0.001
	K	5.81 ± 0.21	4.7 ± 0.13	20.5	<0.001
	Ca	4.73 ± 0.31	3.92 ± 0.16	5.5	0.023
	Mg	1.89 ± 0.1	1.37 ± 0.05	26.8	<0.001