



# Drought Deteriorates the N Stoichiometry of Biomass Production in European Beech Saplings Under Global Change

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Continuous nitrogen (N) deposition has raised concerns that temperate forest ecosystems shift from N limitation to progressing phosphorus (P) limitation under global change. According to the multiple resource limitation theory, this will not only influence P economy, but also reduce N uptake and use efficiencies of trees such that growth is equally limited by N and P. We used different global change scenarios including summer drought to test the prediction of this hypothesis for the N economy of European beech (*Fagus sylvatica* L.) saplings. Our study demonstrates that the N uptake efficiency (NUptakeE) of beech adjusts to soil N availability and stabilizes leaf and fine root N concentrations. By contrast, both efficiencies of N use were curtailed by increasing P limitation under elevated soil N. The photosynthetic N use efficiency (PNUE) was serially reduced with decreasing soil P availability and increasing foliar N:P ratios, while the N use efficiency (NUE) decreased with increasing fine root N:P ratios. Soil drought induced relative P deficiency alike and reduced NUptakeE, PNUE, and NUE independent from the soil N:P ratios. We conclude that not only N deposition but also increasing summer droughts might affect N:P ratios, thereby inducing P imbalances and affecting the N economy of European beech saplings under global change.

**Keywords:** drought, *Fagus sylvatica*, nitrogen deposition, phosphorus limitation, uptake efficiency, use efficiency

## INTRODUCTION

The net primary production of temperate forests is primarily limited by N availability (LeBauer and Treseder, 2008). In recent decades, N availability has rapidly increased in most areas of the globe due to increased N deposition from fossil fuel combustion and intensive agriculture (Galloway et al., 2008). Increased N availability changes nutrient stoichiometry, i.e., leads to rises in the N:P ratio of ecosystems (Peñuelas et al., 2013). As a result, the degree of relative P deficiency increases and potentially shifts temperate forests from N limitation to P limitation (Talkner et al., 2015; Sardans et al., 2016; Hedwall et al., 2017).

According to co-limitation hypothesis, N acquisition and use efficiencies depend on P availability and vice versa (Ågren et al., 2012). According to this theory, P deficiency

will increase P uptake efficiency (PU<sub>takeE</sub>, that is the P content in recent biomass relative to the amount of available soil P; Fujita et al., 2010), but simultaneously may also reduce N uptake efficiency (NU<sub>takeE</sub>, that is the N content in recent biomass relative to the amount of soil N) and N use efficiency (NUE, that is the increment in biomass for a given increase in biomass N). Yet there is currently no mechanistic understanding if (at all) plants achieve simultaneous co-limitation by N and P via modifications in root uptake kinetics (Rothstein et al., 2000) or via changes in root morphology and biomass partitioning (Zhang et al., 2018) or both.

Mycorrhizal symbiosis plays a major role in the P acquisition (Ferlian et al., 2018; Raven et al., 2018) and (to some extent) in the N acquisition of plants (Gobert and Plassard, 2008; Pena and Polle, 2014). Ectomycorrhizal fungi (ECMF) species are functionally diverse and vary in their influence on N acquisition (Pena and Polle, 2014), which makes the ECMF community composition an important factor for nutrient acquisition. In addition, the degree of mycorrhizal colonization affects the portion of mycorrhizal control on resource acquisition. Yet mycorrhizal control may greatly differ between P and N acquisition: low mobility of orthophosphates causes depletion zones around roots and may raise the importance of ECMF hyphae for P access beyond the root depletion zone, while this effect seems less important for highly mobile nitrate.

Nutrient availability also depends on water availability (Gessler et al., 2017). In dry soil, nutrient transport to roots through mass flow and diffusion is slowed down, which can induce edaphic N limitation and reduce the N uptake capacity of trees even in N-rich soil. Soil drought impairs soil microbes and can shift the mycorrhizal community composition with potential consequences for the nutrient uptake of the host tree (Leberecht et al., 2016). This is likely the case in severely dry soil in which the allocation of photosynthates to belowground structures is inadequate (Ruehr et al., 2009). Finally, drought can also decrease root vitality (Burton et al., 1998; Huang et al., 2005; Köhler et al., 2018) and induce fine root dieback (Meier and Leuschner, 2008), which reduces the active root surface area for nutrient uptake. However, the dependence of nutrient stoichiometry and nutrient co-limitation of important temperate forest trees on soil drought as expected under global change are not well understood (Korell et al., 2019).

In previous research, we examined the consequences of different global change scenarios (i.e., elevated temperature, N deposition, reduced soil moisture, and P availability) for P uptake and use efficiencies of European beech saplings (Köhler et al., 2018). We demonstrated that P uptake efficiency of beech in dry soils significantly decreased with decreasing ECMF  $\alpha$  diversity (i.e., the Shannon-Weaver index based on the total number of ECMF species of individual beech sapling), whereas the P use efficiency was negatively related to soil P availability. In the related current study, we aimed to investigate the effect of global change on the N economy of European beech saplings in a four-factorial climate-chamber experiment that simulated the expected conditions for Central Europe. We hypothesized that (H1) beech achieves co-limitation by N and P under increasing soil N availability since NU<sub>takeE</sub> and NUE decrease,

(H2) increasing ECMF colonization rates and diversity increase NU<sub>takeE</sub>, and (H3) summer drought strongly interferes with the acclimation of NU<sub>takeE</sub> of beech to soil N availability and leads to shifts in the N:P stoichiometry and decreases in PNUE and NUE.

## MATERIALS AND METHODS

### Plant Material and Experimental Set-Up

The present study uses the same provenances and experimental set-up as the previous work on the effect of global change on the composition of ECMF communities and the consequences for P uptake and use efficiencies of beech trees (Köhler et al., 2018). Briefly, the experiment was conducted with 2 year old European beech (*Fagus sylvatica* L.) saplings that originated from the 2012 tree masting of a mature beech forest in northwest Germany (N 52° 50', E 10° 19'). The forest site was located on Pleistocene fluvioglacial sandy deposits with relatively low water storage capacity (79 mm 120 cm<sup>-1</sup>) at a mean annual precipitation of 816 mm and with a mean annual temperature of 8.7°C (for the period 1981–2010; climate data were provided by Deutscher Wetterdienst and were corrected for altitude; cf. Knutzen et al., 2017). At this forest site, adult beech trees showed on average a fungal colonization rate of 75% (Hertel et al., 2013) and had comparably species poor ECMF communities with, on average, nine ECMF species in adult trees (Zavišić et al., 2016) and three to six ECMF species in beech saplings (Leberecht et al., 2016). Excavated beech seedlings and their associated indigenous ECMF communities were cultivated in a sterilized 2:1 sand:peat mixture outdoors for one year in the Botanical Garden Göttingen, Central Germany. In May 2014, beech saplings were planted in 1.5-l pots filled with sieved and homogenized sandy mineral topsoil from the provenance, which contained the indigenous ECMF community. Thus, we aimed for maximizing the ECMF colonization rate of the beech saplings. Saplings of similar size (mean shoot height 20 cm, thirteen leaves) were arranged in a randomized array in a greenhouse and were kept well-watered.

In July 2014, we established a full factorial experiment with four factors (two temperature  $\times$  three soil moisture  $\times$  two N supply  $\times$  three P supply levels; 36 treatments in total;  $n$  = eight replicates per treatment; see **Supplementary Table S1**). We simulated a gradient from current environmental conditions to the expected future global change conditions (elevated temperature and N deposition and reduced soil moisture and P availability): we established (i) ambient (T1; 18/12°C day/night) and elevated temperature conditions (T2; 22/16°C) in two separate climate chambers. Furthermore, we set-up (ii) three soil moisture levels an ambient, well-watered (M3; 90% field capacity (FC); 32% soil water content (SWC); v/v), a moderately moist (M2; 60% FC; 21% SWC), and a drought treatment (M1; 30% FC; 11% SWC). The field capacity of the sandy soil used in the experiment was determined according to Naeth et al. (1991). Water loss by evapotranspiration was replaced every second day by watering the pots to their target weight. We established (iii) three P availability levels, a high (P3; 0.69 mg P kg<sup>-1</sup>), a moderate (P2; 0.35 mg P kg<sup>-1</sup>), and a low P supply treatment (P1;

0.17 mg P kg<sup>-1</sup>), by adding biweekly NaH<sub>2</sub>PO<sub>4</sub> as component of the Hoagland solution. Finally, we simulated (iv) ambient (N1; 3.9 mg N pot<sup>-1</sup> growing season<sup>-1</sup>, representing 8.5 kg N ha<sup>-1</sup> year<sup>-1</sup>) and elevated N deposition (N2; 7.8 mg N pot<sup>-1</sup> growing season<sup>-1</sup>, representing 17 kg N ha<sup>-1</sup> year<sup>-1</sup>), estimated on the basis of the current N deposition rates occurring in deciduous forests of Northern Germany (Schaap et al., 2018). N was added biweekly as NH<sub>4</sub>NO<sub>3</sub> as component of the Hoagland solution. Our fertilization regime led to six N:P treatments: relative P availability decreased from N1P3 (0.69 mg P; N:P 5.6 g g<sup>-1</sup>) to N2P3 (0.69 mg P; N:P 11.3 g g<sup>-1</sup>), N1P2 (0.35 mg P; N:P 11.3 g g<sup>-1</sup>), N2P2 (0.35 mg P; N:P 22.6 g g<sup>-1</sup>), N1P1 (0.17 mg P; N:P 22.6 g g<sup>-1</sup>), and N2P1 (0.17 mg P; N:P 45.2 g g<sup>-1</sup>). Beech saplings were randomly assigned to these experimental treatments, positioned in a randomized design in two highly controllable walk-in climate chambers (York, Johnson Control Systems, Essen, Germany), and re-randomized biweekly for two growing seasons. During the non-growing season 2014/2015 (i.e., November 2014–March 2015) temperatures were reduced to 10/8°C day/night, the day length from 14 to 7.5 h, and photosynthetic photon flux density from 190 to 30 μmol photons m<sup>-2</sup> s<sup>-1</sup>, while drought and fertilization treatments were paused. All conditions were brought back to the experimental conditions in April 2015 and maintained until the harvest of the experiment in September 2015. In total, we simulated two subsequent growing seasons with altered climatic and nutrient conditions for a total of 456 days.

## Photosynthetic Capacity

At the end of June 2015, leaf gas exchange measurements were conducted for saplings grown at ambient and elevated temperature, well-watered and dry soil conditions, high and low P availability, and ambient and elevated N availability; each treatment combination being replicated five times ( $n = 80$  saplings). Measurements were conducted on one canopy leaf per plant with an infrared CO<sub>2</sub> analyzer (LI-6400; LI-COR Biosciences, Lincoln, NE, United States) during the midday hours. We measured the photosynthetic capacity ( $A_{\max}$ ; μmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>) at high photosynthetically active radiation (PAR: 1,500 μmol m<sup>-2</sup> s<sup>-1</sup>; see **Supplementary Table S2**). Saturating light conditions were previously determined from light curves measured for a subset of plants. During the measurements, average leaf temperature was 18.2°C (T1) and 22.1°C (T2), relative humidity 61.3% (T1) and 56.2% (T2), vapor pressure deficit 9.6 hPa (T1) and 13.9 hPa (T2), flow rate 400.03 μmol s<sup>-1</sup> (T1) and 400.1 μmol s<sup>-1</sup> (T2), block temperature 15.0°C (T1) and 18.6°C (T2) and CO<sub>2</sub> concentrations were ambient (390 μmol CO<sub>2</sub> mol<sup>-1</sup> air). We calculated total C assimilation per plant (in μmol C h<sup>-1</sup>) by multiplying  $A_{\max}$  with total leaf area, which was determined from the product of specific leaf area (SLA in m<sup>2</sup> kg<sup>-1</sup>; measured by using a flat-bed scanner and the computer program WinFOLIA 2014c; Régent Instruments Inc., Canada) and total dry biomass of green leaves. Photosynthetic N use efficiency (PNUE) was calculated according to  $PNUE = A_{\max} \cdot SLA / N_{\text{Leaves}}$  (in μmol CO<sub>2</sub> g<sup>-1</sup> N s<sup>-1</sup>), where  $N_{\text{Leaves}}$  is the foliar N concentration (cf. Li et al., 2012).

## Above and Belowground Biomass and ECMF Colonization

All beech saplings were divided into senescent leaves, green leaves, shoots, coarse roots (diameter > 2 mm), and fine roots (diameter ≤ 2 mm) in mid-September 2015. For each sapling, we selected one representative, carefully washed, intact fine root section, transferred it to 5% glycerol solution, and kept it frozen at -18°C for subsequent analysis of the mycorrhizal colonization rate (in %;  $n =$  eight replicates per treatment). Fine root tips were counted ( $n = 85,327$ ) and the ECMF morphotypes characterized (as per Agerer, 1987–2012). ECMF colonization rate was calculated as the percentage of the number of ECMF root tips in all vital root tips (see **Supplementary Table S2**). Root vitality was calculated as the percentage of vital root tips in all analyzed fine root tips (in %). Ten to twenty tips of each distinct morphotype were collected and used for molecular identification of fungal species (cf. Köhler et al., 2018). To describe ECMF diversity, we calculated the Shannon-Weaver index (Shannon and Weaver, 1949) from the relative proportion of an ECMF species in the total number of ECMF species per individual beech saplings (ECMF α diversity). Root length and root surface area were determined with WinRHIZO 2013e (Régent Instruments Inc., Canada;  $n =$  eight replicates per treatment). Subsequently, all leaves, stems, coarse roots, and fine roots were dried (48 h, 70°C) and weighed (see **Supplementary Table S3**). Specific root area (SRA, in cm<sup>2</sup> g<sup>-1</sup>) and specific root length (SRL, in m g<sup>-1</sup>) were calculated.

## N Uptake and Use Efficiencies

All dried plant compartments were ground with a vibratory disc mill and were analyzed for total C and N content with an elemental analyzer (Vario EL III, elemental, Hanau, Germany). N uptake efficiency (NU<sub>uptakeE</sub>; in g N<sub>DW</sub> g<sup>-1</sup> N<sub>added</sub>) was calculated by dividing the N content in recent biomass, i.e., in fine roots, green and senescent leaves, by its amount added by fertilization during the 2015 growing season ( $n =$  eight replicates per treatment). N use efficiency (NUE, in g DW g<sup>-1</sup> N<sub>DW</sub>) was calculated from the increment in total dry biomass for a given increase in biomass N (cf. Koide, 1991).

## Statistical Analyses

Statistical analyses were conducted with R 3.3.1 (R Development Core Team, 2017). Means and SE were calculated from eight replicates per treatment ( $A_{\max}$ :  $n = 5$ ). Residuals of the models were tested for normal distribution and homogeneity of variances by Shapiro-Wilk tests. The effects of N availability, P availability, temperature, soil moisture, and their interactions (fixed effects) on the response variables NU<sub>uptakeE</sub>, PNUE, NUE, and plant nutrient concentrations (N concentration, C:N ratio and N:P ratio in leaves, fine roots and coarse roots) were analyzed by four-way ANOVAs type III using the *car* package (Fox and Weisberg, 2011). Tukey's honest significant difference *post hoc* tests were conducted to identify significant differences between individual treatments. We calculated Pearson correlations for the relationships between NU<sub>uptakeE</sub>, PNUE, or NUE with root vitality, ECMF colonization rate, ECMF species richness, ECMF

$\alpha$  diversity,  $A_{\max}$ , C assimilation, leaf and root morphology, and plant nutrient concentrations. For significant correlations, we conducted linear regression analyses and tested the significances of the slopes and intercepts as well as of the entire correlation models. The slopes of all significant correlation models were significant as well, while the intercepts were significant in most cases (exceptions: the intercept of the relationships between NUptakeE and fine root C:N ratio and between PNUE and coarse root C:N). For the relationship between NUptakeE and fine root biomass or fine root C:N ratio, pairwise comparisons of the slopes of N1 and N2 were performed using the “lsmmeans” function in the *lsmmeans* R package (Lenth, 2016). We used SAS v 9.3 (SAS Institute Inc., Cary, NC, US) to conduct multiple regression analyses with backward variable elimination to test for significant independent predictors of NUptakeE, NUE, and PNUE. At each elimination step, the variable showing the smallest contribution to a model was deleted until all the variables remaining in the model produced significant *F* statistics even if their significant contribution was only small. Multicollinearity among variables was diagnosed: when (i) the significance of *t*-tests for individual slopes differed from the *F*-test of the model, (ii) pairs of predictor variables were highly correlated ( $r > 0.7$ ), and (iii) collinearity diagnostics were critical (Belsley et al., 1980).

## RESULTS

### Plant N Concentrations and C:N Ratios in Leaves and Fine Roots

Despite the extended gradients in soil N availabilities (from 3.9 to 7.8 mg N kg<sup>-1</sup>) and soil N:P ratios (from 5.6 to 45.2 g g<sup>-1</sup>), N concentrations in beech leaves, fine roots, and coarse roots remained comparably constant across the range of soil nutrient conditions (see **Supplementary Figures S1A,C,E**). The average N concentration of leaves was 15 mg N g<sup>-1</sup> and the average N concentration of fine roots 10 mg N g<sup>-1</sup> at ambient climate conditions. With elevated temperature and reduced soil moisture, N concentrations increased to 19 mg N g<sup>-1</sup> in leaves and to 15 mg N g<sup>-1</sup> in fine roots. Leaf and fine root C concentrations were similar and not influenced by any of the experimental treatments (leaves and fine roots: 0.5 g C g<sup>-1</sup>). Due to the increase in N concentrations with elevated temperature and drought, fine root and foliar C:N ratios significantly decreased from ambient to climate change conditions (fine roots: from 48 to 33 g g<sup>-1</sup>; leaves: from 31 to 25 g g<sup>-1</sup>; see **Supplementary Figures S1B,F**). Similarly, drought also strongly increased coarse root N concentrations by 83% (from 6 to 11 mg N g<sup>-1</sup>; see **Supplementary Figure S1C**), which led to a decrease in the coarse root C:N ratio by about a half (see **Supplementary Figure S1D**).

### Nitrogen Uptake Efficiency

NUptakeE related negatively to soil N availability (**Figure 1A**, see **Table 2** and **Supplementary Table S4**). Elevated soil N availability reduced NUptakeE of beech saplings by almost half (from 9.5 to 4.9 g N<sub>DW</sub> g<sup>-1</sup> N<sub>added</sub>). Elevated temperature slightly increased NUptakeE by 13% (from 6.8 to 7.7 g N<sub>DW</sub> g<sup>-1</sup> N<sub>added</sub>), while

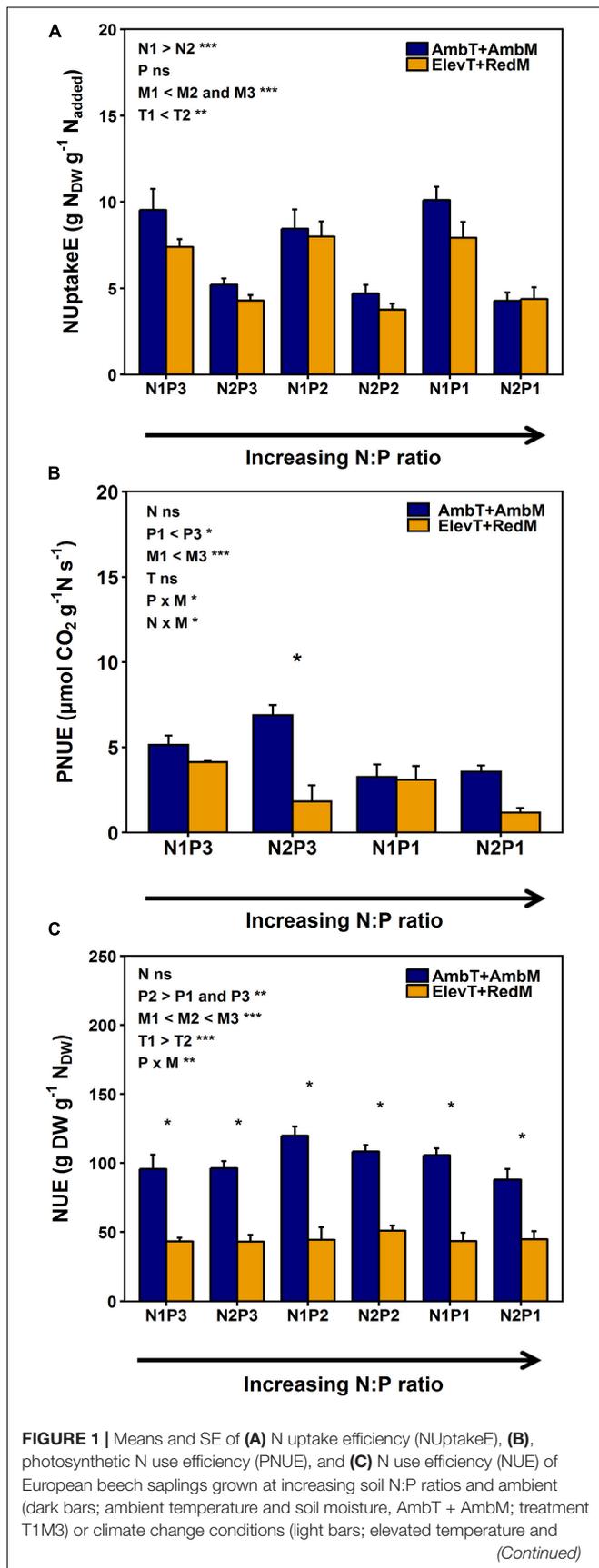
soil drought reduced NUptakeE by 20% (from 7.7 to 6.2 g N<sub>DW</sub> g<sup>-1</sup> N<sub>added</sub>). Soil P availability or the soil N:P ratio had no effect on NUptakeE.

The main intrinsic influence on NUptakeE among the investigated influences had fine root and leaf biomass; that is, the larger the beech sapling and its fine root biomass the more efficient N uptake was ( $R^2 = 0.42$ ; **Tables 1, 3**). The relationship between fine root biomass and NUptakeE differed between soil N availabilities, though. The relationship had a greater intercept and slope at reduced soil N availability (difference significant at  $P < 0.001$  between the slopes of N1 and N2; **Figure 2A**). In contrast to fine root biomass, fine root morphology had no effect on NUptakeE (see **Table 3**). A secondary intrinsic influence on NUptakeE was exerted by root C:N stoichiometry, where NUptakeE increased with the C:N ratio in fine roots and coarse roots (**Figure 2B** and **Table 3**), but with increased scatter of data points around the regression lines ( $R^2 = 0.39$ – $0.41$ ) and non-significant intercepts. The relationship between NUptakeE and the fine root C:N ratio differed between soil N availability and had a greater intercept and slope at reduced soil N availability (**Figure 2B**). However, the slopes of N1 and N2 were only significantly different when single data points were compared ( $P = 0.004$ ; not shown), and not in a comparison of treatment means ( $P = 0.21$ ).

### Nitrogen Use Efficiencies

Surprisingly, PNUE and NUE of the investigated beech saplings were not influenced by soil N availability, but by soil P availability and drought (**Figures 1B,C** and see **Table 2**). PNUE decreased by 45% with a reduction in P availability (from 5.5 to 3.8  $\mu\text{mol CO}_2 \text{ g}^{-1} \text{ N s}^{-1}$  from high to low P availability) and by 57% with soil drought (from 5.4 to 3.4  $\mu\text{mol CO}_2 \text{ g}^{-1} \text{ N s}^{-1}$  from the well-watered to the drought treatment when both temperature treatments were considered; **Figure 1B** and see **Supplementary Table S5**). Hence, there was a significant interaction of P availability and soil moisture on PNUE, which increased with increasing P availability and increasing soil moisture (**Figure 1B**). PNUE declined with decreasing coarse and fine root C:N ratios and increased with fine root N:P ratios (intercept for the relationship between PNUE and coarse root C:N not significant; **Table 1, Figure 3D**, see **Table 3** and **Supplementary Tables S4, S6**), i.e., it was reduced with stoichiometric imbalance and relative deficiency of fine root P. Larger plants with greater and more vital root biomass had higher PNUE (see **Table 3**). Yet the low coefficient of determination in the multiple regression analysis ( $R^2 = 0.25$ ) also indicates additional non-predicted influences on PNUE which did not enter our regression model.

Soil drought had a major impact on NUE (decrease by half from 102 to 53 g DW g<sup>-1</sup> N<sub>DW</sub> from the well-watered to the drought treatment when both temperature treatments were considered; **Figure 1C** and see **Supplementary Table S5**). Further, NUE decreased with an increase in temperature (from 88 to 79 g DW g<sup>-1</sup> N<sub>DW</sub> from ambient to elevated temperature) and at limiting soil P availability (from 91 to 78 g DW g<sup>-1</sup> N<sub>DW</sub> from moderate to low P availability; see **Supplementary Table S5**). Next to these extrinsic influences, the C:N ratios in fine

**FIGURE 1 |** Continued

reduced soil moisture, ElevT + RedM; treatment T2M1) in climate chambers. The associated tables show the results of four-factorial ANOVAs and *post hoc* multiple comparisons according to Tukey for all experimental treatments ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ; ns, not significant;  $n =$  eight replicates per treatment for NUptakeE and NUE;  $n =$  five replicates per treatment for PNUE). Asterisks indicate significant differences between ambient and climate change conditions ( $*P < 0.05$ ). N supply: N1, ambient; N2, elevated. P supply: P1, low; P2, moderate; P3, high. Temperature: T1, ambient; T2, elevated. Soil moisture: M1, drought; M2, moderately moist; M3, well-watered; see also **Supplementary Table S1**.

and coarse roots or the degree of relative N deficiency strongly increased NUE of saplings (**Figure 3A** and **Tables 1, 3**). The larger the leaf biomass of beech saplings was (and the higher the ECM colonization rate tended to be) the more efficient was their N use (**Table 1** and **Figure 3B**). NUE was also significantly enhanced by increasing ECMF species richness and ECMF diversity (see **Table 3**). When all experimental treatments were considered, it appeared that ECMF colonization rate related negatively to N concentrations in leaves, coarse roots, and fine roots (see **Supplementary Table S7**), and positively to the corresponding C:N ratios (but with increased scatter of data points around the regression lines:  $R^2 = 0.23$ ; **Figure 3C**).

## DISCUSSION

### Reduced N Uptake Efficiency Compensates for Elevated N Availability

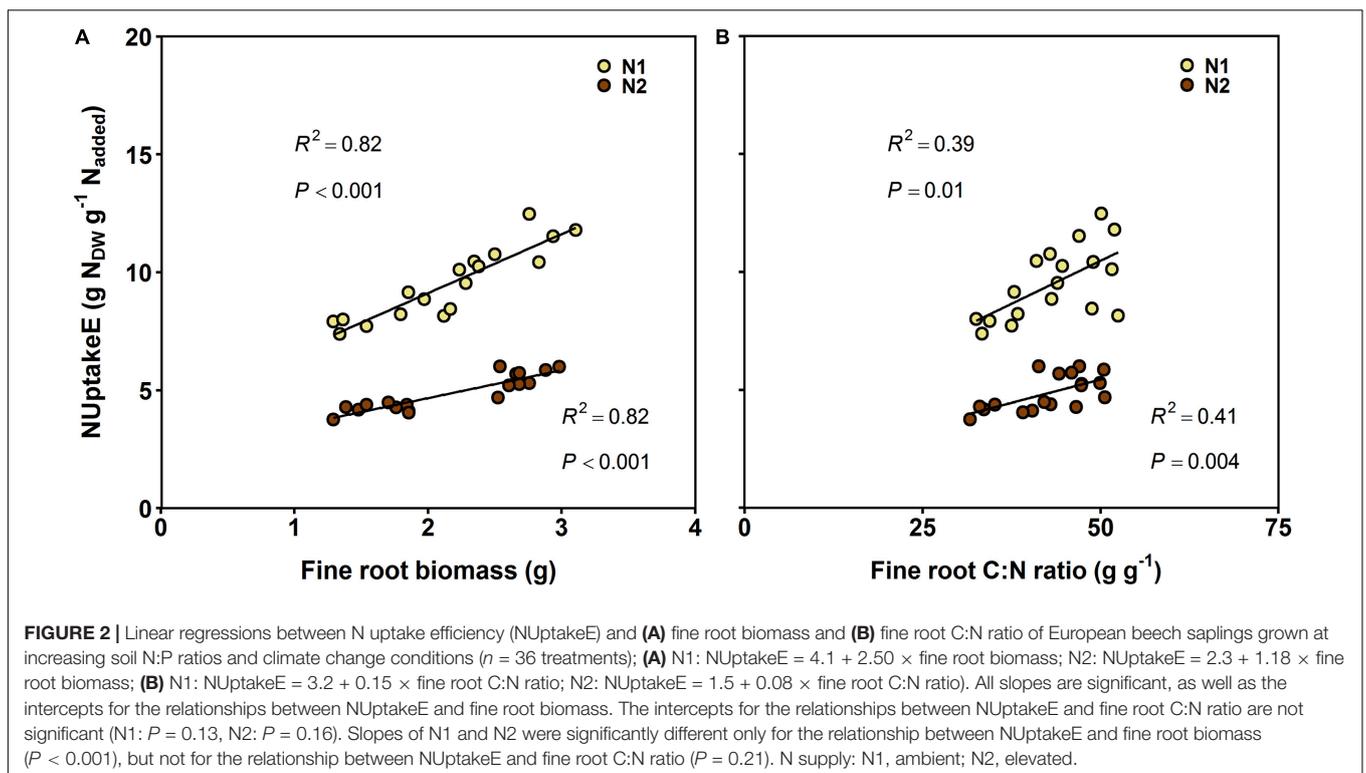
Elevated N deposition and summer droughts in the course of global change are assumed to affect nutrient stoichiometry (Vitousek et al., 2010; Peñuelas et al., 2013; Sardans et al., 2016), but it remains unclear to what degree plants counteract these changes by adjustments of the uptake efficiencies of individual nutrients. Here we demonstrate that European beech saplings respond to an increase in N availability by sufficient adjustments of NUptakeE (**Figure 1A**), with the consequence that tissue N concentrations and NUE remained comparably constant across the range of soil N availabilities and soil N:P ratios (**Figure 1C** and **Supplementary Figure S1**) but at the cost of a major tissue N:P stoichiometric change (**Supplementary Table S6**).

The capacity for N acquisition can be adjusted to N availability by changes in biomass partitioning, shifts in morphological or physiological traits, or by alterations at the cellular level (Kraiser et al., 2011) such as switching from low to high affinity nitrogen uptake (Gan et al., 2016). Previous fertilization experiments or N deposition studies showed that increases in N availability enhance total plant productivity, but they decrease fine root biomass partitioning (Nadelhoffer, 2000; Corrêa et al., 2011) and shift the ECMF community to nitrophilic taxa or even decrease ECMF colonization (Kjøller et al., 2012; de Witte et al., 2017). Yet in our fertilization study which simulated a naturally low to moderate N range, fine root biomass partitioning, ECMF colonization rate, and root morphology were unaffected by soil N availability (see also Köhler et al., 2018). A closer inspection of the regression between NUptakeE and fine root biomass illustrates that the

**TABLE 1** | Three independent multiple regression analyses on the effects of multiple predictor plant traits on N uptake efficiency (NUptakeE), photosynthetic N use efficiency (PNUE), or N use efficiency (NUE).

Dependent variable	Model			Selected Predictor variables	F	P
	R <sup>2</sup>	P				
NUptakeE	0.42	<0.001	+	Fine root biomass	48.2	<0.001
			+	Leaf biomass	11.3	0.001
			–	Fine root C:N	13.5	0.002
PNUE	0.25	<0.001	–	Leaf N:P	13.2	<0.001
			+	Plant biomass	5.8	0.02
NUE	0.84	<0.001	+	Fine root C:N	91.7	<0.001
			+	Coarse root C:N	76.3	<0.001
			+	Leaf biomass	60.4	<0.001
			–	Fine root biomass	31.9	<0.001
			–	Fine root N:P	14.5	<0.001
			+	ECMF colonization rate	7.7	0.01

Tested predictor plant traits were leaf and fine root biomass, coarse root C:N and N:P, leaf and fine root N, leaf and fine root C:N and N:P, root vitality, ECMF colonization rate, ECMF species richness, and ECMF  $\alpha$  diversity. Values given are the determination coefficient R<sup>2</sup> and the probability of error P for the model and the F-value and probability of error P for the selected predictors. The “+” or “–” signs at the predictor variables indicate positive or negative relationships.



relationship has similar coefficient of determination and is shifted to almost half the slope and to only a fraction of the intercept at elevated N availability. Theoretically, this bisection of the relationship can be caused by either a categorical shift in root architecture and functioning or by alterations in the membrane structure and transporter activity at elevated N availability. Plants can curtail NUptakeE by alterations in N uptake. Under high N availability, they exhibit a lower density and substrate affinity of nitrate transporters (Kraiser et al., 2011; Castro-Rodríguez et al., 2017). This shift in the relationship between NUptakeE and

fine root biomass can stabilize tissue N at a given concentration optimal for plant functions and growth, as it was the case in the investigated European beech saplings.

The reduction of NUptakeE of beech by half when soil N availability was doubled reveals the negative relationship between NUptakeE and soil N availability. At the same time, the plant N:P ratio increased with increasing soil N:P, even though at a lower rate, which points at metastable tissue P concentrations and rising relative P limitation under elevated soil N:P. This rise in relative P limitation was attenuated in the investigated

**TABLE 2** | Four-factorial ANOVAs (type III SS) on the effects of N availability (N), P availability (P), temperature (T), soil moisture (M), and their interactions on the variance of N uptake efficiency (NUptakeE), photosynthetic N use efficiency (PNUE), and N use efficiency (NUE) of European beech saplings grown at increasing soil N:P ratios and climate change.

	NUptakeE		PNUE		NUE	
	F	P	F	P	F	P
N	<b>299.4</b>	<b>&lt;0.001</b>	1.7	0.20	1.4	0.24
P	0.5	0.60	<b>7.0</b>	<b>&lt;0.05</b>	<b>7.0</b>	<b>&lt;0.01</b>
T	<b>7.6</b>	<b>&lt;0.01</b>	1.3	0.26	<b>13.2</b>	<b>&lt;0.001</b>
M	<b>14.6</b>	<b>&lt;0.001</b>	<b>17.3</b>	<b>&lt;0.001</b>	<b>143.7</b>	<b>&lt;0.001</b>
P × N	1.5	0.22	0.1	0.77	0.3	0.77
P × T	0.8	0.46	0.0	0.87	0.2	0.79
N × T	0.5	0.49	3.3	0.07	0.0	0.91
P × M	0.7	0.61	<b>6.2</b>	<b>&lt;0.05</b>	<b>3.9</b>	<b>&lt;0.01</b>
N × M	1.1	0.32	<b>4.0</b>	<b>&lt;0.05</b>	2.5	0.08
P × N × T	1.0	0.36	0.0	0.89	0.0	0.97
P × N × M	1.1	0.38	0.7	0.39	1.2	0.32

Efficiencies were square root transformed to resemble normality and homogeneity of variances. Given are F-values and probabilities of error P. Significant effects are indicated by bold letters ( $n =$  eight replicates per treatment for NUptakeE and NUE;  $n =$  five replicates per treatment for PNUE).

beech saplings by an increase in PUptakeE (Köhler et al., 2018), but not to the extent that no rise in tissue N:P occurred. Theoretically, plants may counteract an increase in tissue N:P ratio by both a (costly) increase in PUptakeE or a (cheaper) further restriction of NUptakeE when soil P is increasingly limiting. In our investigation the adjustment of NUptakeE was unresponsive to the availability of soil P and merely a function of N availability and fine root biomass. It seems that European beech saplings are only able to adjust their NUptakeE to the availability of N, but not to the availability of P. Biochemically dependent co-limitation (as defined by Saito et al., 2008) does not appear to play a role for N uptake.

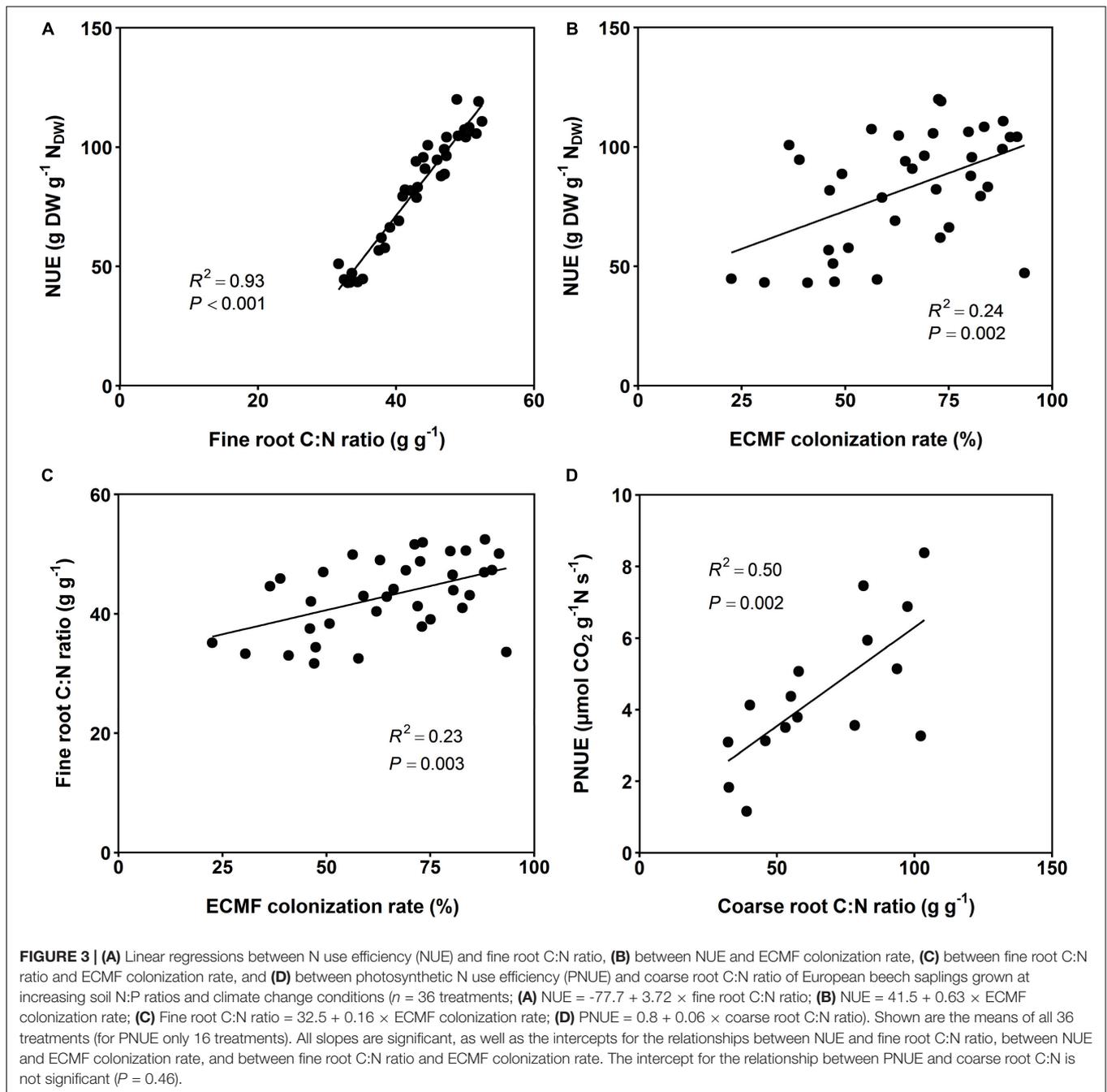
## Photosynthetic N Use Efficiency Is Curtailed by Serial Limitation and Drought

In contrast to previous studies which focused on aboveground NUE in mature forests (e.g., Vitousek, 1982; Aerts and Chapin, 1999; Tateno et al., 2017), PNUE and whole-plant NUE of beech saplings were not influenced by soil N availability but were related to soil P availability instead (Figures 1B,C and Table 2). This points at stoichiometric constraints in PNUE and NUE, either from P or C limitation (Blanes et al., 2013). PNUE decreased with increasing P deficiency and decreasing  $A_{max}$  at high leaf N:P ratios. At a closer inspection it becomes clear that a response of PNUE to an increase in soil N availability occurs only after soil P availability increases (in the control climate; Figure 1B). This is an example of serially linked nutrient limitation as defined by Ågren et al. (2012), where the plant response to a second nutrient depends on the prior addition of another limiting resource. In the case of PNUE of beech, this response was synergistic and super-additive when both nutrients were added, but it was also

dependent on the order of their addition. Serially linked nutrient limitation links to Liebig's law of the minimum (Von Liebig, 1855) and indicates no strict co-limitation of PNUE by P and N. As a consequence of this serial limitation, PNUE of beech will probably not respond to N deposition as long as soil P continues to be increasingly limiting for plants (Talkner et al., 2015; Sardans et al., 2016; Hedwall et al., 2017).

Soil drought reduced PNUE by more than one third and had an even stronger effect at high soil N availability, which emphasizes the essential and overriding role of C limitation in decreasing PNUE. At the same time, foliar N increased in dry soil independent from soil N availability or the soil N:P ratio while the foliar C:N ratio decreased. Increased leaf N contents with drought have previously been observed in plants around the world (Sardans et al., 2017). Assimilation under drought can be optimized by increased leaf N contents to sustain the photosynthetic capacity (Farquhar et al., 2002). Increased leaf N contents with drought were associated with better stress adaptation from cellular membrane stability, lower osmotic potentials, reduced lipid peroxidation, and increased osmoprotectant (e.g., proline) content (Ashraf and Foolad, 2007). In our study, the increase in absolute and relative leaf N content under drought was not a consequence of greater availability and uptake of soil N. Rather, it was probably related to missing investment opportunities of N when drought is leading to reductions in stomatal opening width and consequently in photosynthetic C assimilation, i.e., to stronger drought effect on biomass production than on N to a stronger drought effect. More N is then stored in vacuoles and not used for growth, which leads to a decrease in the foliar C:N ratio. Similarly, the N concentration of absorptive foraging fine roots of mature beech trees increased during a severe summer drought while their C:N ratio decreased, which was probably consequence of a relatively high proportion of ephemeral roots with a high N concentration (Nikolova et al., 2020). Nevertheless, even if this increase in foliar N in our study may have counteracted to some extent the most severe drought stress, it was not sufficient to avoid any stomatal closing and decreases in photosynthetic capacity in drought-exposed beech saplings.

In dry soil, photosynthetic C assimilation of beech saplings was also limited by rising foliar N:P ratios and relative P limitation (Köhler et al., 2018). The factorial addition of P and soil moisture revealed a super-additive independent co-limitation (as defined by Harpole et al., 2011) by the two factors on PNUE, yet with a dominating effect exerted by soil drought and a subordinate effect by P deficiency (see Supplementary Figure S2). Previously, we have demonstrated that drought reduces the P uptake efficiency of beech from reductions in ECMF diversity and in the complementarity of P uptake by ECMF species (Köhler et al., 2018; see also Kreuzwieser and Gessler, 2010; Pena et al., 2010; Zavišić et al., 2016). This drought-induced P limitation probably also establishes the secondary role of P deficiency on PNUE. Limitations in plant P are reducing the ATP supply and thus the amount, regeneration, and specific activity of Rubisco (Reich et al., 2009; Yang et al., 2016), which decreases photosynthetic C assimilation and PNUE in addition to the direct stomatal control from soil drought. The observed



significant decrease in PNUE in P-limited beech is in accordance with the results obtained from other tree species (Reich et al., 2009; Gan et al., 2016). Yet the dominating influence of soil moisture on PNUE will leave the response of European beech to P deficiency strongly dependent on future precipitation patterns.

### Soil Drought Disturbs Nutrient Stoichiometry as a Result of Reductions in ECMF Diversity

NUE decreased with decreasing fine and coarse root C:N ratios and decreasing leaf biomass, i.e., when less photosynthates were

available for beech growth. This decrease in NUE occurred at both high and low soil P availabilities (that is NUE is, when all experimental treatments are considered, significantly higher at P2 than at P1 and P3, as shown in the four-factorial ANOVA of Figure 1C) probably due to different reasons: the decrease in NUE with low soil P availability can be a consequence of a decrease in photosynthetic capacity and a reduction in PNUE from P limitation, as described above. The decrease in NUE of beech at high soil P can be explained by a reduction of photosynthetic P use efficiency when P is excessively supplied (cf. Lambers et al., 2012; Hidaka

**TABLE 3** | Pearson's correlations between N uptake efficiency (NUptakeE), photosynthetic N use efficiency (PNUE), and N use efficiency (NUE) with plant biomass, leaf and root morphology, ectomycorrhizal fungal (ECMF) community, photosynthetic capacity, nutrient concentrations, and nutrient ratios of European beech saplings grown at increasing soil N:P ratios and climate change.

	NUptakeE	PNUE	NUE
Plant biomass	<b>0.39*</b>	<b>0.72**</b>	<b>0.90***</b>
Leaf biomass	<b>0.41*</b>	<b>0.63**</b>	<b>0.88***</b>
$A_{\max}$	0.27	<b>0.94***</b>	<b>0.64**</b>
SLA	-0.12	-0.40	<b>-0.66**</b>
Total C assimilation	0.49	<b>0.88***</b>	<b>0.87***</b>
$C_{\text{leaves}}$	-0.16	<b>-0.69**</b>	<b>-0.59***</b>
$N_{\text{leaves}}$	-0.24	<b>-0.70**</b>	<b>-0.89***</b>
$P_{\text{leaves}}$	0.07	<b>0.67**</b>	0.33
$C:N_{\text{leaves}}$	0.25	<b>0.69**</b>	<b>0.86***</b>
$N:P_{\text{leaves}}$	-0.07	<b>-0.64**</b>	<b>-0.43**</b>
Coarse root biomass	<b>0.39*</b>	<b>0.70**</b>	<b>0.92***</b>
$C_{\text{coarse roots}}$	-0.03	-0.34	-0.06
$N_{\text{coarse roots}}$	-0.25	<b>-0.65**</b>	<b>-0.92***</b>
$P_{\text{coarse roots}}$	-0.06	0.31	-0.14
$C:N_{\text{coarse roots}}$	<b>0.33*</b>	<b>0.71**</b>	<b>0.93***</b>
$N:P_{\text{coarse roots}}$	0.02	-0.49	-0.24
Fine root biomass	<b>0.37*</b>	<b>0.77***</b>	<b>0.84***</b>
SRL	0.01	-0.24	<b>-0.36*</b>
SRA	0.02	0.11	-0.26
Root vitality	0.23	<b>0.54*</b>	<b>0.52**</b>
ECMF colonization rate	0.25	0.04	<b>0.49**</b>
ECMF species richness	0.16	0.10	<b>0.39*</b>
ECMF $\alpha$ diversity	0.14	0.18	<b>0.38*</b>
$C_{\text{fine roots}}$	0.23	<b>-0.51*</b>	0.08
$N_{\text{fine roots}}$	-0.33	<b>-0.63**</b>	<b>-0.96***</b>
$P_{\text{fine roots}}$	-0.03	0.44	0.01
$C:N_{\text{fine roots}}$	0.32	<b>0.58*</b>	<b>0.96***</b>
$N:P_{\text{fine roots}}$	-0.08	<b>-0.60*</b>	<b>-0.36*</b>

Given are the correlation coefficients  $R$  and the probabilities of error  $P$  (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ;  $n = 36$  treatments). Significant correlations are indicated by bold letters. SLA, specific leaf area; SRA, specific root area; SRL, specific root length.

and Kitayama, 2013; Köhler et al., 2018). More P is then invested in non-photosynthetic membrane structures (Lambers et al., 2012) or accumulated in vacuoles (Elser et al., 2010; Gan et al., 2016) when surplus P cannot be used for growth. Previous studies found a decrease in NUE with increasing N availability in different deciduous tree species (e.g., Gan et al., 2016; Leberecht et al., 2016), but did not report on a decrease in NUE when the P supply was high. The observed close relationship of both PNUE and NUE with soil P availability in our study emphasizes the important role of the N:P stoichiometry for energy-dependent metabolic processes and, thus, beech NUE and growth.

The negative drought effect on the efficiencies for N uptake and N use intensified from NUptakeE (-20%) to PNUE (-37%) and NUE (-48%) (Supplementary Table S5); that is downstream its metabolic pathway from uptake to photosynthetic processing and biomass production in beech saplings. In addition, drought

adjusted the different levels of PNUE and NUE to a similar, low level across the range of soil N:P ratios (Figure 1). At the same time, tissue N concentrations increased under drought (Supplementary Figure S1). The constant NUE with increasing tissue N in dry soil points at a counteracting effect by nutrient imbalances and P limitation (Supplementary Table S6), which are causing growth declines of beech saplings. In a comparison of the nutrient uptake efficiencies, drought had an even stronger effect on PUptakeE (as reported in Köhler et al., 2018) than on NUptakeE (cf. Dirks et al., 2019). Drought strongly decreased PUptakeE of beech saplings as a consequence of decreased ECMF colonization rate and the loss of ECMF with long-distance exploration. Soil exploration is more important in P uptake than in N uptake, since soil P occurs mostly in adsorbed, undissolved forms and diffuses very slowly (Lambers et al., 2008). Due to the loss in soil exploration by ECMF in dry soil, the negative drought effect was three times higher for PUptakeE than for NUptakeE and led to a shift in uptake stoichiometry. In our multiple regression analysis, ECMF colonization rate was identified as a secondary influence on beech NUE. This likely reflects the indirect role of hampered ECMF colonization rate on NUE through decreases in PUptakeE and increases in P deficiency, which decreases PNUE (as described above) and beech sapling growth from deteriorated N:P stoichiometry. As a consequence of these water limitations and nutrient imbalances, leaf and coarse root biomass and NUE strongly decreased while the root:shoot biomass ratio increased in beech saplings exposed to drought. It seems that soil drought has the potential to disturb the adjustment of plant stoichiometry to elevated N deposition.

## CONCLUSION

We conclude that elevated N availability aggravates growth reductions from nutrient imbalances and summer droughts in European beech saplings. Notably, the direct negative effect of experimental drought on N uptake efficiency of European beech saplings intensifies downstream the metabolic pathway (that is from uptake to use) from additional limitations by P and C. Decreases in the ECMF colonization rate and ECMF diversity reduce P uptake efficiency in dry soil, leading to reductions in PNUE. Therefore, N use efficiency in dry soil strongly decreases from a combination of limited P uptake efficiency (and N uptake efficiency) and reduced photosynthetic C fixation leading to deteriorated nutrient stoichiometry and reduced beech sapling growth when exposed to experimental global change conditions. If such an effect also occurs in European beech saplings under natural conditions will have to be tested in subsequent field trials.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

IM conceived of and designed the study and obtained the funding. JK and NY performed the experiments and collected the data. JK and IM analyzed and interpreted the data. IM, JK, AP, and RP wrote the manuscript. All authors approved the final version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/ffgc.2021.647360/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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