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LETTER

Soil Nitrogen Supply Exerts Largest Influence on Leaf Nitrogen in Environments with the Greatest Leaf Nitrogen Demand

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ABSTRACT

Accurately representing the relationships between nitrogen supply and photosynthesis is crucial for reliably predicting carbon–nitrogen cycle coupling in Earth System Models (ESMs). Most ESMs assume positive correlations amongst soil nitrogen supply, leaf nitrogen content, and photosynthetic capacity. However, leaf photosynthetic nitrogen demand may influence the leaf nitrogen response to soil nitrogen supply; thus, responses to nitrogen supply are expected to be the largest in environments where demand is the greatest. Using a nutrient addition experiment replicated across 26 sites spanning four continents, we demonstrated that climate variables were stronger predictors of leaf nitrogen content than soil nutrient supply. Leaf nitrogen increased more strongly with soil nitrogen supply in regions with the highest theoretical leaf nitrogen demand, increasing more in colder and drier environments than warmer and wetter environments. Thus, leaf nitrogen responses to nitrogen supply are primarily influenced by climatic gradients in photosynthetic nitrogen demand, an insight that could improve ESM predictions.

1 | Introduction

In our modern world, where human activities have nearly doubled bio-available inorganic nitrogen (Ackerman, Millet, and Chen 2019; Galloway et al. 2008; Vitousek et al. 1997), what dictates the response of leaf nitrogen to soil nitrogen supply? This question is crucial for accurately predicting carbon fluxes between the atmosphere and terrestrial biosphere, given the intimate coupling of the carbon and nitrogen cycles (Hungate et al. 2003; Thornton et al. 2007). Because nitrogen is a major constituent of proteins regulating photosynthetic processes (Evans 1989; Evans and Clarke 2019; Evans and Seemann 1989), leaf nitrogen and photosynthetic capacity are often positively correlated (Evans 1989; Kattge et al. 2009; Walker et al. 2014) and leaf nitrogen is often used to predict photosynthetic capacity in Earth System Models (ESMs) (Smith and Dukes 2013; Wieder et al. 2019). Although positive correlations between leaf nitrogen content and soil nitrogen supply have been observed (Firn et al. 2019; Li et al. 2020; Liang et al. 2020), assessing the drivers that shape the magnitude of leaf nitrogen response to soil nitrogen supply poses challenges. Plant nitrogen allocation is temporally and spatially variable (Onoda et al. 2017) and leaf nitrogen content (on both a mass and area basis) can be influenced by the demand for nitrogen to support leaf metabolism, leaf traits such as leaf mass per unit area, and nitrogen demand for growth. Furthermore, both demands could be impacted by climatic conditions (Smith et al. 2019; Stocker et al. 2020), biochemical pathways involved in photosynthesis (C_3 vs. C_4) (Ghannoum, Evans, and Von Caemmerer 2010; Simpson et al. 2020), nutrient acquisition strategy (e.g., symbiotic association with N_2-fixing bacteria, hereafter, N_2-fixers vs. non-fixers) (Adams et al. 2016), and interactions with other soil nutrients (e.g., phosphorus and potassium) (Harpole et al. 2017).

Based on the assumption that plants will attempt to minimise costs of resource uptake and use based on their specific growing environments, eco-evolutionary optimality principles (Franklin et al. 2020; Harrison et al. 2021; Wright, Reich, and Westoby 2003), which are grounded on optimal coordination (Chen et al. 1993; Maire et al. 2015) and least-cost hypotheses (Wright, Reich, and Westoby 2003), can predict leaf nitrogen demand from climatic variables. Many studies have demonstrated that leaf nitrogen demand for photosynthesis, particularly for ribulose-1,5-biphosphate (RuBP) carboxylase oxygenase (Rubisco) carboxylation (V_{cmax}), is predominantly determined by climatic factors rather than by soil nitrogen supply (Dong et al. 2017; Onoda et al. 2017; Paillassa et al. 2020; Peng, Bloomfield, and Prentice 2020; Peng et al. 2021; Prentice et al. 2014; Smith et al. 2019; Smith and Keenan 2020; Stocker et al. 2020; Wang et al. 2017; Waring, Perkowski, and Smith 2023; Westerband et al. 2023; Wright, Reich, and Westoby 2003). However, some V_{cmax} variability also appears to be impacted by belowground resources (Paillassa et al. 2020; Smith et al. 2019; Yan et al. 2024).

Theory predicts that, in arid locations, elevated atmospheric aridity increases potential transpiration per leaf area and the associated maintenance costs (Prentice et al. 2014). To minimise these costs, plants will reduce stomatal conductance while simultaneously increasing nitrogen use and allocation to photosynthetic enzymes to optimise carbon uptake at the lowest summed resource use cost (Westerband et al. 2023; Wright, Reich, and Westoby 2003). Consequently, reduced stomatal conductance results in a reduced internal-to-ambient CO_2 ratio (C_i/C_a; denoted as χ) (Cornwell et al. 2018; Dong et al. 2020; Medlyn et al. 2011; Prentice et al. 2011). Therefore, leaf nitrogen content is expected to correlate negatively with χ (Figure 1, path a), and the leaf nitrogen response to soil nitrogen supply is likely to be more pronounced under arid conditions compared to wetter conditions.

Similarly, in cold climates, low temperatures decrease the reaction rates of Rubisco (Ali et al. 2015; Dong et al. 2017; Hinojo-Hinojo et al. 2018; Rogers et al. 2017; Smith and Dukes 2018; Wang et al. 2020), and soil nitrogen availability (McGroddy, Daufresne, and Hedin 2004; Reich and Oleksyn 2004). To optimise photosynthesis while mitigating this decline in enzymatic activity, coupled with soil nitrogen limitations, leaves are likely to increase the synthesis of Rubisco. Consequently, leaf nitrogen content is expected to negatively correlate with temperature (Figure 1, path b), and the leaf nitrogen response to soil nitrogen supply is anticipated to be more pronounced as climatic temperatures decrease.

Lastly, in high-irradiance environments, leaves are expected to optimise light resource utilisation (Borer et al. 2013; Dong et al. 2017; Niinemets, Keenan, and Hallik 2015; Paillassa et al. 2020; Smith et al. 2019) by allocating more nitrogen to Rubisco, resulting in a positive correlation between leaf nitrogen content and irradiance (Figure 1, path c).

Leaf nitrogen demand can also be influenced by a plant's N_2-fixation capacity. For instance, N_2-fixers have higher leaf nitrogen content than non-fixers (Adams et al. 2016; Vergutz et al. 2012) because they obtain nitrogen from the atmosphere. Consequently, N_2-fixers are expected to be less responsive to soil nitrogen supply than non-fixers.

C_4 plants are expected to have lower leaf nitrogen demand than C_3 plants. This is because C_4 plants have reduced photorespiration and higher nitrogen use efficiency compared to C_3 plants (Ghannoum, Evans, and Von Caemmerer 2010; Jones 2010). C_4 plants can achieve a higher photosynthetic rate with less nitrogen than C_3 plants due to their carbon concentrating mechanism (Jones 2010; Vogan and Sage 2011). Consequently, C_4 leaves are expected to demonstrate lower leaf nitrogen demand and a weaker responsiveness to soil nitrogen supply.

Moreover, phosphorus, potassium, and microelements can affect both photosynthetic foliar nitrogen demand and plant nitrogen demand for growth. Drawing from our understanding of how phosphorus limitations affect leaf metabolism (Bloomfield, Farquhar, and Lloyd 2014; Crous et al. 2017; Ellsworth et al. 2015, 2022; Plaxton and Tran 2011; Reich, Oleksyn, and Wright 2009; Warren and Adams 2002), it is conceivable that phosphorus supply, resulting in elevated leaf phosphorus, may primarily enhance the maximum electron transport for RuBP regeneration (J_{max}) and, to a lesser extent, the V_{cmax} (Ellsworth et al. 2022). This enhancement could lead to an increased leaf nitrogen demand (Luo et al. 2021; Warren and Adams 2002). Consequently, a greater increase in leaf nitrogen content could be anticipated following soil nitrogen supply in phosphorus-fertilised locations.

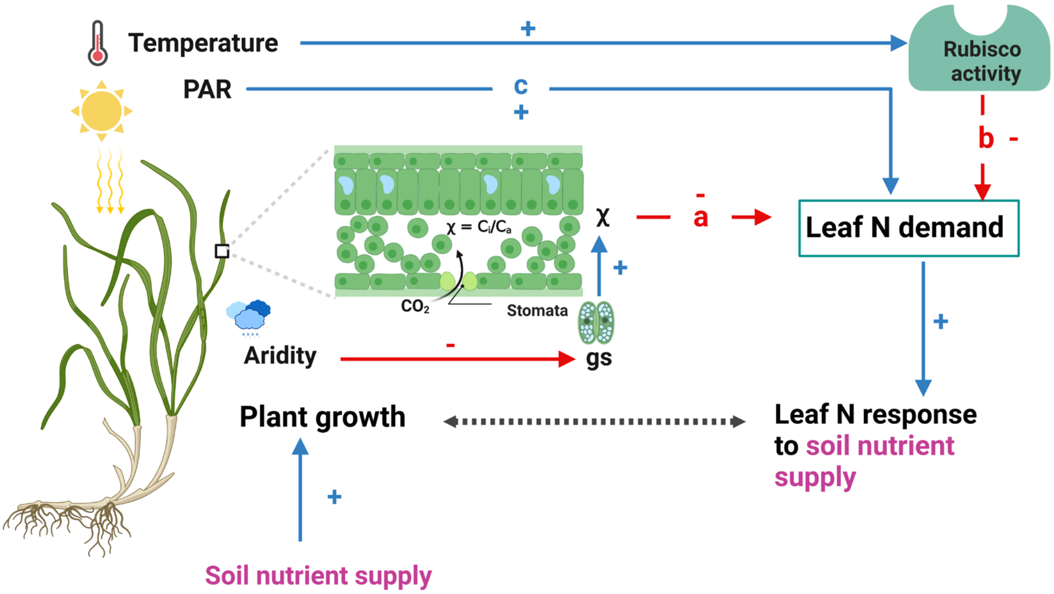

FIGURE 1 | Conceptual illustration of the anticipated impacts of climatic factors on leaf nitrogen demand and subsequent responses to soil nitrogen supply. An increase in aridity, represented by the ratio of mean annual precipitation to mean annual potential evapotranspiration, is anticipated to induce stomatal conductance reduction, leading to a decrease in the ratio of intracellular to extracellular CO_2 (χ) through a positive relationship between stomatal conductance and χ. This decrease in χ is expected to trigger an upregulation of Rubisco to compensate for the decline in χ, consequently leading to an increase in leaf nitrogen demand as indicated by a negative relationship between χ and nitrogen demand (path a). Additionally, temperature has a positive relationship with Rubisco activity. There is a predicted negative relationship between Rubisco activity and leaf nitrogen demand, as leaves are expected to enhance the synthesis of photosynthetic enzymes (path b). Conversely, an increase in incoming radiation is anticipated to enhance demand for Rubisco to maximise light utilisation, leading to an increase in leaf nitrogen demand (path c). Elevated leaf nitrogen demand is projected to enhance the leaf nitrogen response to soil nitrogen addition. Ultimately, the addition of soil nutrients (nitrogen, phosphorus, and potassium) is expected to augment aboveground biomass and the overall nitrogen demand of the entire plant. This study aims to validate these expectations and evaluate the intricate interactions between soil nutrient supply, leaf nitrogen demand, and whole-plant growth responses under varying climatic conditions.

All these possible effects on leaf nitrogen demand and subsequent leaf nitrogen response to soil nutrient supply could interact with the whole plant nitrogen demand for growth. Some studies suggest that changes in soil nitrogen supply are reflected in alterations in biomass rather than in leaf nitrogen (Fay et al. 2015; Feng et al. 2023; Harpole et al. 2017; LeBauer and Treseder 2008; Li et al. 2020). If plants use added nitrogen to construct new organs, a dilution effect may occur, resulting in attenuated leaf nitrogen responses to soil nitrogen supply.

To assess the drivers of leaf nitrogen response to soil nutrient supply at species level, we used data from the Nutrient Network (NutNet), a globally distributed grassland nutrient addition experimental network encompassing diverse climates, with added nutrients experimentally increasing soil nutrient supply.

We hypothesised that leaf nitrogen responses to soil nitrogen supply would depend on leaf photosynthetic nitrogen demand, in conjunction with whole-plant nitrogen demand (Figure 1). Specifically, we expected that:

1. Climate drivers and leaf traits serve as stronger predictors of leaf nitrogen content than soil nitrogen supply, causing leaf nitrogen content to exhibit a negative relationship with temperature and leaf internal to ambient CO_2 ratio (χ), but a positive relationship with light availability.
2. Leaf nitrogen content will be greater in N_2-fixers and C_3 plants compared to non-fixers and C_4 plants.
3. Leaf nitrogen demand will dictate the leaf nitrogen response to soil nitrogen supply, such that high aridity (Figure 1, path a), low temperature (Figure 1, path b), and high light availability (Figure 1, path c) will increase leaf nitrogen response to soil nitrogen supply. Additionally, phosphorus and potassium supplementations are expected to increase leaf nitrogen demand and, therefore, leaf nitrogen response to soil nitrogen supply.
4. The anticipated increase in leaf nitrogen response to soil nitrogen supply with increasing leaf nitrogen demand is expected to be mitigated when added nitrogen enhance biomass production.

2 | Methods

2.1 | Leaf Traits, Biomass, and Species Cover Data Acquisition

Data were collected from 26 grassland sites within the NutNet experiment (Borer et al. 2014). These sites cover a broad climatic gradient and are distributed across diverse biogeographical zones (Figure S1). Each site follows a complete randomised block design with at least three blocks, eight nutrient treatments per block, and three replicates per treatment, yielding

24 experimental units ($N=24$, 5×5 m plots). Nutrient treatments followed a standardised protocol, involving nitrogen (N) (10 g N m^{-2} $year^{-1}$, as timed-release urea), phosphorus (P) (10 g P m^{-2} $year^{-1}$, as triple super phosphate), and potassium (K) (10 g K m^{-2} $year^{-1}$, as potassium sulphate). A macro-and micronutrient mix, including iron, sulphur, magnesium, manganese, copper, zinc, boron, molybdenum, and calcium, was added to all K plots once in the first year at a rate of 100 g m^{-2} $year^{-1}$, as such, we refer to these plots as $K_{+\mu}$. Each nutrient was applied at two levels (control, added), crossed in a full-factorial design (Control, N, P, $K_{+\mu}$, NP, $NK_{+\mu}$, $PK_{+\mu}$, and $NPK_{+\mu}$). The oldest sites commenced nutrient supply in 2008 (Table S2). Further details in (Borer et al. 2014).

For this analysis, we paired a published leaf trait dataset from this experimental platform (Firn et al. 2019) with biomass and species areal cover generated by the NutNet (Borer et al. 2014).

The leaf trait dataset (Firn et al. 2019) included leaf nutrient content, leaf carbon isotopes, leaf area, and leaf dry matter. Leaf area and leaf dry matter were used to calculate LMA. Leaf trait data were conducted 3–4 years after the initiation of nutrient addition, corresponding to the peak biomass period for the most abundant species in each plot (between one to nine species per plot were selected depending on the site and plot within site). For each selected species, five fully developed leaves from five mature individuals were chosen and combined to measure the specified variables. Further details in Firn et al. (2019).

The data characterising biomass and species areal cover included plot-level peak biomass of living aboveground tissue, assessed annually for the same plots and sites where leaf traits were measured. Briefly, in each plot, a 2.5×2.5 m subplot was divided into four 1×1 m permanent sub-subplots. Plant biomass sampling included clipping of all plants rooted within two 0.1 m^2 strips, totaling 0.2 m^2. The collected live biomass was subsequently dried to a constant mass and weighed. A visual estimation of the areal cover percentage for each species was conducted for every plot within a separate 1×1 m sub-subplot. Per species live biomass was estimated by multiplying this percentage by the total live biomass for the plot (Ladouceur et al. 2022).

Finally, we assessed whether each species has the capacity to form symbiosis with N_2-fixing bacteria or employs a C_3 or C_4 photosynthetic pathway based on past literature for each species.

2.2 | Leaf Trait Calculations

The variables used in this study are outlined in Table S1. To evaluate our hypotheses, we conducted analyses on all samples with data pertaining to leaf nitrogen content on a mass basis (N_{mass}; g_N g_{leaf}^{-1}), leaf nitrogen content on area basis (N_{area}; g_N m_{leaf}^{-2}), leaf mass per unit area (LMA, g_{leaf} m_{leaf}^{-2}), leaf carbon isotope discrimination ($\delta^{13}C_{leaf}$ (‰)), and aboveground biomass AGB (g_{plant} m_{soil}^{-2}).

N_{area} was calculated from N_{mass} using LMA:

$$N_{area} = N_{mass} \times \mathrm{LMA} \tag{1}$$

We calculated the ratio of intracellular to extracellular CO_2 (χ; Pa Pa^{-1}) from $\delta^{13}C_{leaf}$ following (Farquhar, Ehleringer, and Hubic 1989) (Text S1). Values of χ less than 0.1 and greater than 0.95 were excluded from our analysis, as these extremes could represent outliers arising from uncertain parameters. This refinement led to a dataset comprising 1752 data-points from 196 species across 26 sites (Figure S1).

2.3 | Climate Data

The climatic variables considered included the mean annual growing season temperature (T_g;°C) and incoming photosynthetically active radiation (PAR; μmol m_{soil}^{2} s^{-1}), averaged monthly over the period 1901–2015. The growing season was operationally defined as the months with mean temperatures exceeding 0°C. Data for T_g and cloudiness were extracted from the Climatic Research Unit (CRU TS3.24.01) (Harris et al. 2014) at a resolution of 0.5°. Cloudiness data were used to calculate PAR using the SPLASH model (Davis et al. 2017).

To assess the aridity at each site, we extracted the moisture index (hereafter MI) for the period 1970–2000 at a spatial resolution of 30 arcminutes from the global aridity database (Global-AI_PET_v3) (Zomer, Xu, and Trabucco 2022). MI is the ratio of mean annual precipitation to potential evapotranspiration, reflecting both precipitation and water loss factors like temperature, radiation, and wind. Low MI signifies arid sites with minimal precipitation or significant water loss. The climatic variables for each site are summarised in Table S2.

2.4 | Data Analysis

2.4.1 | Leaf Nitrogen Content

To assess the drivers of N_{mass} and N_{area} and their respective importance, we employed a linear mixed-effects model. The dependent variables were N_{mass} or N_{area}, and fixed effects included climatic variables (T_g, PAR), χ, LMA, as continuous effects, and nutrient treatment variables (soil N, P, and $K_{+\mu}$ treatments, and their interactions), photosynthetic pathway (two levels: C_3 or C_4), and N_2-fixation (two levels: N_2-fixers or non-fixers) as categorical effects. Categorical random intercept terms included species identity, species identity nested within the site, and species identity nested within the site and block. N_{mass}, N_{area}, and LMA were natural log-transformed to meet normal distribution assumptions and minimise skewness. To evaluate the potential effect of multicollinearity between predictors on the interpretation of the results, we calculated variance inflation factors (VIFs) for each independent fixed effect (Davis et al. 1986). We considered multicollinearity to be a concern when VIF values were above 5 (Kutner et al. 2004).

2.4.2 | Percentage Changes in Leaf Nitrogen Content and Biomass

To analyse the responses of N_{mass}, N_{area}, and AGB to soil nitrogen addition, we calculated the percentage change in N_{mass} (ΔN_{mass}; %), N_{area} (ΔN_{area}; %), and AGB (ΔAGB; %) from the ambient soil

N plots to the added soil N plots, considering each species within each P and $K_{+\mu}$ treatment within each block within each site. Specifically, ΔN_{mass} from any given P and $K_{+\mu}$ treatment within each block at every site was computed as:

$$\Delta N_{\text{mass}} = \left(\left(N_{\text{mass,trt+N}} - N_{\text{mass,trt−N}}\right) / N_{\text{mass,trt−N}}\right) \times 100 \quad (2)$$

where $N_{\text{mass,trt+N}}$ was the N_{mass} in a given P and $K_{+\mu}$ treatment within a given block that received nitrogen and $N_{\text{mass,trt-N}}$ was the N_{mass} in the same P and $K_{+\mu}$ treatment within the same block that did not receive nitrogen. The same procedure was applied to ΔN_{area} and ΔAGB. To eliminate outliers, we applied a conservative median absolute deviation method as described by Leys et al. (2013), excluding percent change values that were three times higher or lower than the median absolute deviation.

We fit linear mixed-effects models with ΔN_{mass} and ΔN_{area} as the dependent variables. Climatic variables (T_g, *PAR*, and MI) were included as continuous fixed effects, while soil treatment variables (soil P and $K_{+\mu}$, along with their interactions), photosynthetic pathway, and N_2-fixation, were included as categorical fixed effects. Categorical random intercept terms consisted of species identity, species identity nested within site, and species identity nested within site and block.

To investigate the interaction between ΔAGB and ΔN_{mass} and to disentangle the direct and indirect effects of climatic variables, soil nutrient treatments, photosynthetic pathway, and N_2-fixation on ΔN_{mass} through its covariance with ΔAGB, we used a structural equation model (SEM). Firstly, ΔAGB was predicted from climatic variables (T_g, *PAR*, and MI), soil treatment variables, photosynthetic pathway, and N_2-fixation. Subsequently, ΔN_{mass} was predicted from ΔAGB and all predictors of ΔAGB to isolate indirect and direct effects, respectively, of climate and soil on ΔN_{mass}. The effect of T_g on MI was also introduced to account for the connection between these two predictors. Species identity was included for all paths as a random intercept term.

All linear mixed-effect models were fit using the "*lmer*" package (Bates 2018) in R version 4.3.1(R Core Team 2023). We used Wald's chi-squared tests to test the statistical significance of each fixed effect term in the models using "car" package (Fox and Weisberg 2019) in R. Post hoc analyses were conducted using the "*emmeans*" package (Lenth et al. 2024) in R. For all models, relative importance of each variable was calculated as the R^2 partitioned by averaging over orders (Lindeman, Merenda, and Gold 1979) using "*calc.relimp*" function in the "*relaimpo*" package in R (Grömping 2006). The structural equation model was fit using the 'PiecewiseSEM' package (Lefcheck 2016) using functions from the '*lme*' package (Bates 2018) in R.

All data and code used for these analyses are available at: https://doi.org/10.5281/zenodo.13952400.

3 | Results

3.1 | Drivers of N_{mass} and N_{area} and Their Relative Importance

Leaf nitrogen content on a mass basis (N_{mass}) was 20.14% greater in plots that received supplemental nitrogen than in those that did not receive supplemental nitrogen ($p < 0.001$; Table 1, Figure 2, and Table S3). Soil nitrogen supply had a more

TABLE 1 | Regression coefficients for linear mixed-effects model with N_{mass} as the dependent variable and soil treatments, climate variables, leaf mass on area basis, photosynthetic pathway, and N_2-fixation as fixed effects.[a]

	df	Slope	*p*	Relative Importance	VIF
Soil N	1	—	***<0.001***	5.3%	4
Soil P	1	—	0.81	2.01%	3.9
Soil $K_{+\mu}$	1	—	0.77	1.7%	4
T_g	1	-0.0263 ± 0.006	***<0.001***	8.74%	1.2
PAR	1	0.0004 ± 0.0001	**0.003**	18.22%	1
ln *LMA*	1	-0.0554 ± 0.0112	***<0.001***	9.04%	1
χ	1	-0.2433 ± 0.1144	**0.03**	25.18%	1.1
N_2 fixation	1	—	***<0.001***	9.62%	1
Photosynthetic pathway (C_3/C_4)	1	—	***<0.001***	6.5%	1.3
Soil N × Soil P	1	—	**0.001**	1%	5.9
Soil N × Soil $K_{+\mu}$	1	—	0.56	0.95%	5.9
Soil P × Soil $K_{+\mu}$	1	—	0.95	0.53%	5.9
Soil N × Soil P × Soil $K_{+\mu}$	1	—	0.56	0.37%	6.8

Note: Sample size is 1432. Number of species = 178. *Key*: Soil N (soil nitrogen supply), Soil P (soil phosphorous supply), and Soil $K_{+\mu}$ (soil potassium and micronutrient supply) are categorical (ambient or added). T_g, *PAR*, *LMA* and χ are continuous. N_2 fixation (yes or no) and photosynthetic pathway (C_3 or C_4) are categorical. Slopes are only included for continuous fixed effects. The relative importance of each variable was assessed using Lindeman, Merenda, and Gold (lmg) variance decomposition in *R*, quantifying their contribution to total amount of variation explained by the model (R^2 partitioned through an averaging process over multiple orders). The VIF of each variable represents the variance inflation factor. The total model conditional R^2 was 0.83, and marginal R^2 was 0.46.

[a] $p < 0.05$ are bolded and $p < 0.001$ are italicised.

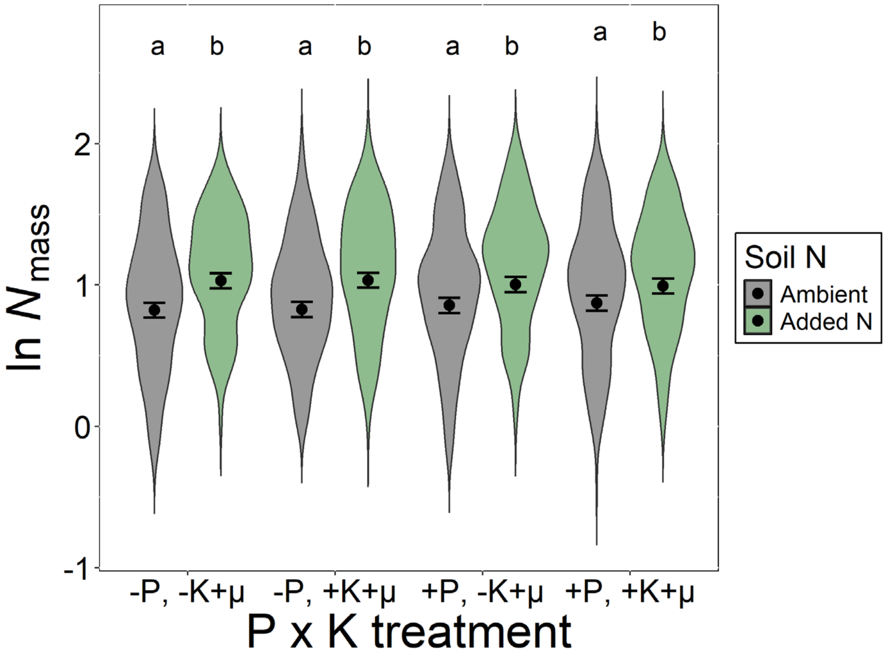

FIGURE 2 | Log-transformed N_{mass} at the species level, depicted under different soil nitrogen (N) conditions, including ambient soil nitrogen (grey violins) and conditions with added soil nitrogen (green violins) in various treatment plots: Those without added phosphorus (P) or potassium ($K_{+\mu}$) (−P, −K + μ), plots without added phosphorus but with added potassium (−P, +$K_{+\mu}$), plots with added phosphorus but without added potassium (+P, −$K_{+\mu}$), and plots receiving both phosphorus and potassium (+P, +$K_{+\mu}$). Points and error bars correspond to the mean values and standard errors calculated by the linear mixed effects model applied. The lettering above each box indicates groupings based on post hoc Tukey's tests, where different letters indicate statistically different groups at $\alpha = 0.05$ across all groups shown.

pronounced positive impact on N_{mass} in plots without phosphorus (25.16% increase) compared to plots with phosphorus (15.35% increase; soil N by P interaction: $p < 0.001$; Figure 2, Table 1, and Table S3). Furthermore, N_2-fixers exhibited 70.29% greater N_{mass} than non-fixers ($p < 0.001$; Table 1 and Table S4), and C_3 species displayed 88.92% greater N_{mass} than C_4 plant species ($p < 0.001$; Table 1 and Table S4). Similar trends were observed for N_{area} (Figure S2, Tables S5–S7).

Although the impacts of soil nitrogen treatments on N_{mass} and N_{area} were statistically significant, the drivers related to climate and LMA emerged as stronger predictors of leaf nitrogen content. Notably, these factors exhibited greater relative importance in the model compared to soil nutrient supply, as illustrated by the tree maps shown in Figure 3 and Figure S3. For N_{mass}, χ (25.18%), *PAR* (18.22%), LMA (9.04%), and T_g (8.74%) combined to explain 61.2% of N_{mass} variability (Figure 3 and Table 1). Additionally, N_2-fixation (9.62%) and photosynthetic pathway (6.5%) combined (16.12%) were more important than soil nutrient treatments and their interactions (11.86%) in the N_{mass} model (Figure 3 and Table 1).

For N_{area}, χ (18.72%), *PAR* (6.68%), LMA (45.64%), and T_g (4.84%) combined to explain 75.9% of N_{area} variability (Table S5 and Figure S3). Additionally, N_2-fixation (2.05%) and photosynthetic pathway (5.65%) combined (7.7%) were more important than soil nutrient treatments and their interactions (4.44%) in the N_{area} model.

In both the N_{area} and N_{mass} models, the directionality of the χ (negative), T_g (negative), and *PAR* (positive) slopes (Table 1, Figure 3, Table S5 and Figure S3) aligned with theoretical expectations. All slopes between climatic drivers and leaf nitrogen content were significantly different from zero ($p < 0.05$, Table 1 and Table S5).

3.2 | Drivers of Leaf Nitrogen Response to Nitrogen Supply

As anticipated, there was a significant increase in ΔN_{mass} and ΔN_{area} with decreasing MI and decreasing T_g (Figure 4a,b, Figure S4a,b, Tables S8 and S9). Slopes for both T_g and MI in relation to both ΔN_{mass} and ΔN_{area} were significantly different from zero ($p < 0.05$ in both cases; Tables S8 and S9). However, contrary to our predictions, *PAR* did not show any effect on ΔN_{mass} or ΔN_{area} (Figure 4c and Figure S4c, $p > 0.05$; Tables S8 and S9). There was a significant effect of soil phosphorus treatments on ΔN_{mass} and ΔN_{area} ($p < 0.05$ in both cases), but no significant effect of soil $K_{+\mu}$ treatments or the interaction between phosphorus and $K_{+\mu}$ (Tables S8 and S9). Soil phosphorus supply negatively impacted ΔN_{mass} (44.15% decrease; Figure 4d), and N_2-fixers had a weaker response to soil nitrogen supply (i.e., lower ΔN_{mass}) than non-fixers (Figure 4e and Figure S4e). Photosynthetic pathway did not significantly affect ΔN_{mass} ($p = 0.32$; Table S8) or ΔN_{area} ($p = 0.277$; Table S9).

The SEM (Figure 5) did not reveal any evidence of an impact of ΔAGB on ΔN_{mass}. Instead, a direct and significant negative effect of increasing MI and *Tg* on ΔN_{mass} persisted in the model. Additionally, N_2-fixation and phosphorus supply showed a significantly negative direct effect on ΔN_{mass}. Climatic factors, soil phosphorus and $K_{+\mu}$, photosynthetic pathway, and N_2-fixation did not demonstrate any significant influence on the ΔAGB, except for a positive effect of *PAR* on ΔAGB.

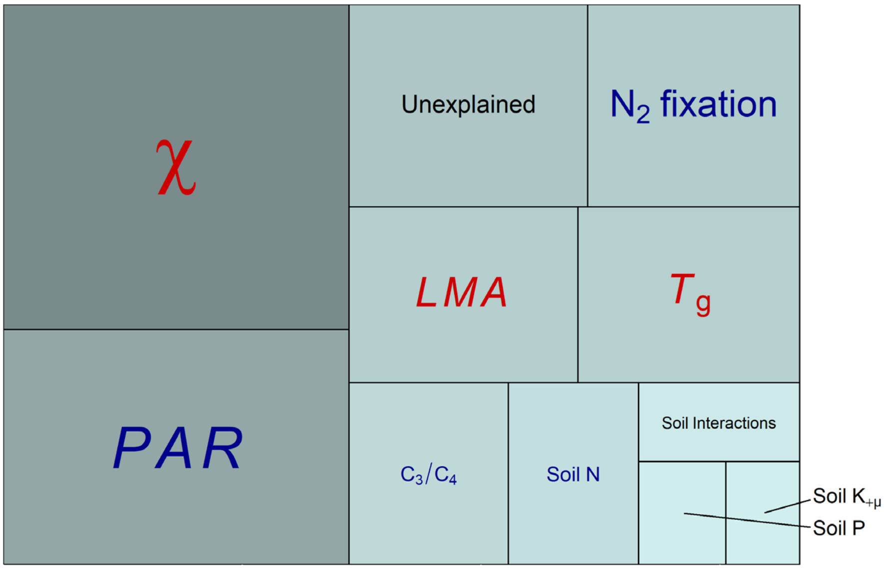

FIGURE 3 | Treemap of relative importance for the linear mixed-effects model with N_{mass} as the dependent variable and soil treatment variables, climate variables, leaf mass per unit area, and species functional types as fixed effects. The area of the tree map represents 100% of the variance in the N_{mass} data. The size and hue of each box is proportional to the relative importance of each factor, with larger and darker boxes indicating greater importance (Table 1). χ = ratio of intracellular to extracellular CO_2 concentration (Pa Pa^{-1}), C_3/C_4 = indicator of whether the plant uses the C_3 or C_4 photosynthetic pathway (categorical; C_3 or C_4), LMA = leaf mass on area basis (g_{leaf} m_{leaf}^{-2}), N_2-fixation = indicator of whether the species is known to associate with symbiotic nitrogen fixing bacteria (categorical; yes or no), *PAR* = growing season photosynthetically active radiation (μmol m^{-2} s^{-1}), Soil K + μ = soil potassium and micronutrient supply (categorical; ambient or added), Soil N = soil nitrogen supply (categorical; ambient or added), Soil P = soil phosphorous supply (categorical; ambient or added), T_g = growing season temperature (°C). Letters in red indicate negative relationships with N_{mass} whereas blue letters indicate positive relationships with N_{mass}.

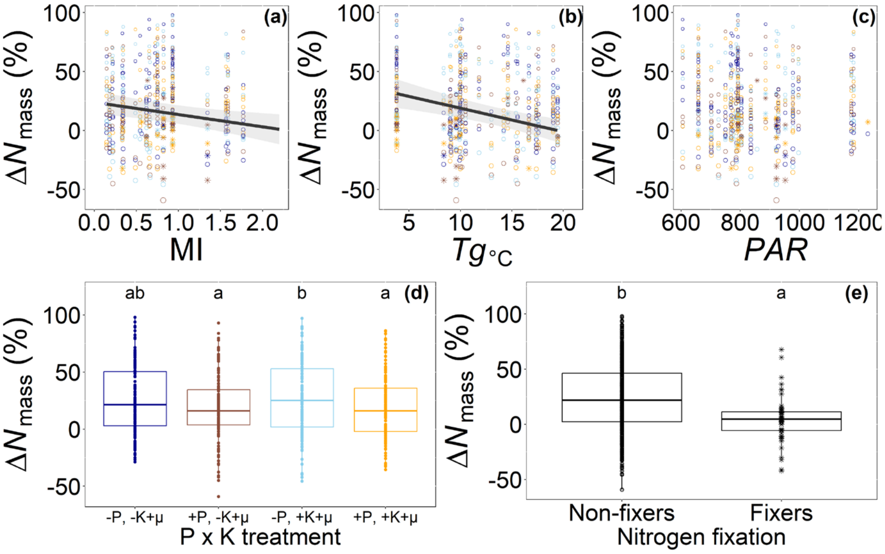

FIGURE 4 | Scatter plots depicting the relationship between (a) ΔN_{mass} and global moisture index (MI), (b) between ΔN_{mass} and growing season temperature (T_g), and (c) between ΔN_{mass} and photosynthetically active radiation (*PAR*) (c). Box plots depicting the (d) variation of ΔN_{mass} depending on P and $K_{+\mu}$ treatments, and (e) N_2-fixation capacity. Significant linear regressions (a and b) are represented by the regression lines derived from a linear mixed-effects model, with ΔN_{mass} as the dependent variable and soil P and $K_{+\mu}$ treatments, MI, T_g, *PAR*, photosynthetic pathways, and N_2-fixation capacity as fixed effects. Shaded regions around the regression lines represent 95% confidence intervals. Larger dots indicate higher ΔN_{mass}. Star dots indicate N_2-fixers, while circle dots indicate non-fixers. Dark blue dots indicate treatments which did not receive P and $K_{+\mu}$, brown dots indicate treatments which received P but not $K_{+\mu}$, blue light indicate treatments which received $K_{+\mu}$ but not P, and orange dots indicate treatments which received P and $K_{+\mu}$. The lettering above each box indicates groupings based on post hoc Tukey's tests, where different letters denote statistically different groups at $\alpha = 0.05$ across all groups shown.

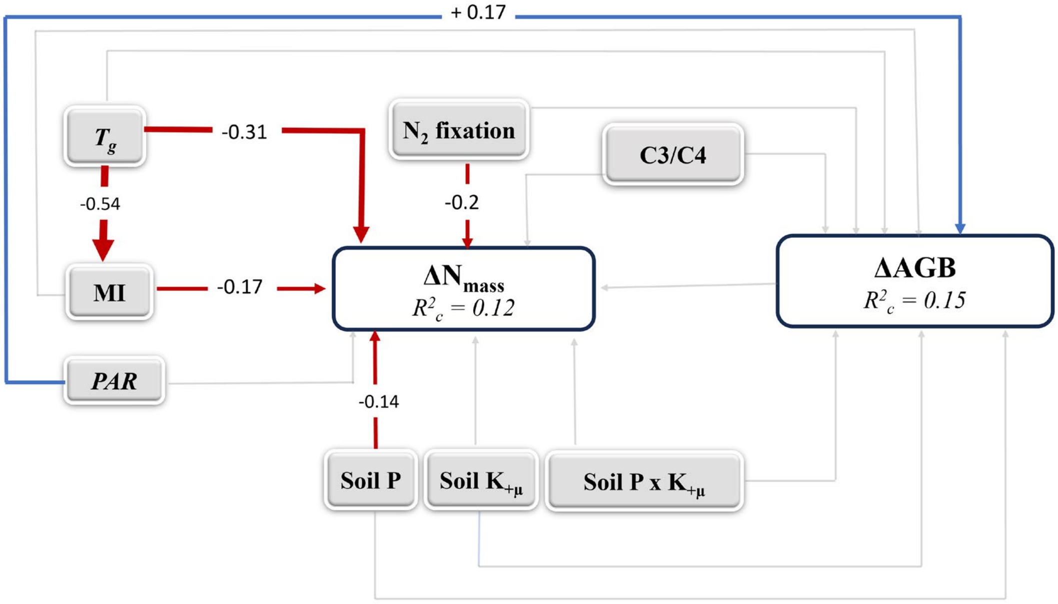

FIGURE 5 | Structural equation model illustrating the interaction between ΔAGB and ΔN_{mass} and disentangling the direct and indirect effects of climatic variables, soil nutrient treatments, photosynthetic pathways, and N_2-fixation on ΔN_{mass} through its covariance with ΔAGB. Path coefficients are depicted as simple standardised regression coefficients. The width of connections indicates estimates of standardised path coefficients, with solid lines denoting significant connections and semi-transparent lines indicating non-significant connections. Negative relationships are depicted by red lines. Positive relationships are depicted by blue lines. R^2 for component models is given in the boxes of response variables and is reported as the conditional R^2 (R^2c) based on the variance of both the fixed and random effects.

4 | Discussion

Predicting leaf nitrogen dynamics is crucial for modelling terrestrial ecosystem responses to global changes. Our findings emphasise the importance of predictable, climate-driven leaf nitrogen demand in explaining leaf nitrogen responses to soil nitrogen supply at the species level. These responses were affected by symbiotic association with N_2-fixing bacteria, and by phosphorus supply, but not by aboveground biomass changes.

4.1 | Leaf Nitrogen Responses to Soil Nitrogen Supply Are Greater Under Arid and Cold Environments

Climate-related variables (T_g, *PAR*), χ, and LMA significantly influenced N_{mass} and N_{area}, explaining 61% and 76% of their variance, respectively. Soil nutrient supply had a lesser impact compared to climate and LMA in explaining leaf nitrogen content variation. Increased N_{area} with soil nitrogen supply was mainly due to elevated N_{mass}, consistent with Firn et al. (2019), who, using a similar grassland nutrient supply dataset, found no impact of soil nutrient addition on LMA. The robust positive relationship between N_{area} and LMA was anticipated since N_{area} was calculated from LMA. Similarly, the negative correlation between N_{mass} and LMA was anticipated due to the inverse relationship between leaf dry matter and nitrogen concentration.

The inverse correlation observed between leaf nitrogen content and χ, coupled with the negative relationship between the percentage change in leaf nitrogen content and MI, confirms that plants maintain elevated leaf nitrogen content and exhibit a heightened demand for leaf nitrogen when stomatal conductance is reduced to maintain light utilisation for photosynthesis (Wright, Reich, and Westoby 2003). These findings align with observational studies (Fan et al. 2023; Luo et al. 2021; Peng et al. 2021; Prentice et al. 2014; Westerband et al. 2023), underscoring the consistent response of plants in adjusting their leaf nitrogen content in response to aridity.

Similarly, the inverse correlation observed between leaf nitrogen content and T_g, coupled with the negative relationship between the percentage change in leaf nitrogen content with soil nitrogen supply and T_g, suggests that decreased enzymatic speed at low temperatures leads to an increased requirement for enzymes necessary to maximise light utilisation, as substantiated in observational studies (Ferreira Domingues et al. 2015; Rogers et al. 2017; Smith et al. 2019; Wang et al. 2020) and temperature manipulation experiments (Smith and Keenan 2020). In fact, V_{cmax} normalised to a standard temperature (commonly 25°C) tends to be higher at cooler sites (Dong et al. 2022; Rogers et al. 2017) and shows a decline with increasing temperature (Dong et al. 2017; Ferreira Domingues et al. 2015; Fürstenau Togashi et al. 2018; Scafaro et al. 2017).

The positive correlation observed between leaf nitrogen content and *PAR* aligns with the relationship between light and plant investment in photosynthetic enzymes (Boardman 1977; Niinemets, Keenan, and Hallik 2015). However, the percentage change in leaf nitrogen content was not found to significantly increase with increasing *PAR*. This could be partially explained by the significant direct positive effects of *PAR* on the percentage change of aboveground biomass (Figure 5), suggesting that greater production of biomass can dilute nitrogen. Additionally, previous studies indicate plants adjust photosynthetic capacity to meet leaf nitrogen demands dictated by light availability irrespective of soil nitrogen levels (Dong et al. 2017; Poorter et al. 2019; Waring, Perkowski, and Smith 2023) with the proportion of leaf nitrogen used for photosynthesis increasing with higher light availability (Waring, Perkowski, and Smith 2023). Finally, the temperature- and aridity-driven response of N_{mass} and N_{area} to soil nutrient supply was not influenced by changes in aboveground biomass (Figure 5), indicating that leaf nitrogen response to soil nutrient supply remains unaffected by increases or decreases in aboveground biomass. In a similar community-level study by Anderson et al. (2018) using the NutNet experiment, the authors found that climate and nutrient availability were strong drivers of community-level nutrient pools, but that this was due more to modifications in community biomass than community nutrient concentrations. This contrasts with our findings and suggests that different processes may operate at the community level-potentially influenced by species compositional turnover that can alter the interaction between leaf nutrient responses at the species level and plant nutrient and biomass responses at the community level.

4.2 | N_2-Fixers and C_3 Species Maintain High Leaf Nitrogen Content due to Their High Leaf Nitrogen Demand

Our findings highlight the significance of photosynthetic pathway and N_2-fixation in predicting both N_{mass} and N_{area}. N_2-fixers demonstrated higher N_{mass} and N_{area} compared to non-fixers, consistent with prior studies (Adams et al. 2016; Vergutz et al. 2012). The greater leaf nitrogen content in N_2-fixers could be understood through the lens of least-cost hypothesis, as N_2-fixers predominate in arid and semi-arid environments where leaf nitrogen demand for photosynthesis is higher and χ is lower, compared to wet environments (Adams et al. 2016; Menge, Wolf, and Funk 2015). Additionally, the expected limited responsiveness of leaf nitrogen content to soil nitrogen supply observed in N_2-fixers is complex, especially considering that N_2-fixation did not affect the percentage change in aboveground biomass in response to nitrogen supply. Elevated soil nitrogen often inhibits symbiotic N_2-fixation (Barron, Purves, and Hedin 2011; Batterman et al. 2013; Hartwig 1998; Perkowski, Waring, and Smith 2021; Sullivan et al. 2014) because the symbiosis becomes more costly than using soil nitrogen as its supply increases (Gutschick 1981). However, some studies suggest that soil nitrogen has no effect on symbiotic N_2-fixation (Drake 2011; Menge et al. 2023). To reconcile these differences, two distinct ways that symbiotic N_2-fixation responds to soil nitrogen have been proposed (Menge, Wolf, and Funk 2015): facultative N_2-fixers adjust fixation after using soil nitrogen, while obligate N_2-fixers maintain fixation regardless of soil nitrogen availability. Obligate N_2-fixers should be stable in consistently nitrogen-limited environments where adjusting symbiosis is costly, while facultative N_2-fixers are stable in nitrogen- fluctuating environments with low adjustment costs. In our experiment, the lack of detailed information on soil nitrogen availability beyond the categorical structure (ambient or added) prevents us from drawing definitive conclusions about the mechanisms driving the reduced responsiveness of leaf nitrogen content in N_2-fixers.

Finally, we found that leaf nitrogen content was higher in C_3 species compared to C_4 species, confirming previous studies (Sage and Pearcy 1987; Simpson et al. 2020; Yuan et al. 2007). C_4 species are known to reduce photorespiration and fix more carbon despite a lower investment in photosynthetic enzymes (Ghannoum, Evans, and Von Caemmerer 2010; Jones 2010) due to their higher CO_2 concentration around Rubisco compared to C_3 species. Consequently, C_4 species may have lower leaf nitrogen demand, explaining their comparatively lower leaf nitrogen content. Surprisingly, the percentage change in leaf nitrogen content in response to soil nutrient supply did not significantly differ between photosynthetic pathways. Since we were examining leaf nitrogen responses to soil nitrogen at the species level, various local ecological processes at the community level (e.g., disturbance, competition, herbivory) could influence species-level responses. Local ecological factors have been shown to modify C3 and C4 grass responses to broad-scale climatic drivers (Griffith et al. 2015). Species compositional shifts in response to soil nutrient supply can alter both aboveground and belowground environments (e.g., light and edaphic resources), potentially impacting the nitrogen response of C3 and C4 plants at species level. Further investigation into how community-level processes interact with climatic factors to shape leaf nitrogen responses in C3 versus C4 plants could improve our ability to accurately model future carbon budget in ESMs.

4.3 | Leaf Nitrogen Response to Soil Nutrient Supply Was Higher Where Phosphorus Was Not Added

Unexpectedly, we observed a lower responsiveness of N_{mass} and N_{area} in phosphorus-addition plots compared to phosphorus-ambient plots, although phosphorus supply is reported to enhance photosynthetic activity (Ellsworth et al. 2022), potentially increasing leaf nitrogen demand. Moreover, this reduced responsiveness of leaf nitrogen content to soil nitrogen supply in phosphorus-addition plots was not explained by increased percentage change in aboveground biomass (Figure 5). However, in a similar grassland nutrient supply dataset, phosphorus addition in nitrogen-ambient plots was shown to increase leaf nitrogen concentration (Firn et al. 2019), indicating enhanced nitrogen uptake and emphasising the role of other nutrient limitations in boosting plant nitrogen uptake. Consequently, the lesser responsiveness of leaf nitrogen content to soil nitrogen supply in phosphorus-addition plots compared to phosphorus-ambient plots in our study may suggest that the leaf nitrogen demand was already met in phosphorus-addition plots. Phosphorus addition alone may have sufficiently stimulated leaf nitrogen uptake, fulfilling the nitrogen requirements for photosynthesis even with ambient soil nitrogen levels. Further research is needed to understand how nitrogen and phosphorus supply interact with climate to affect photosynthesis and growth.

4.4 | Limitations

This study is subject to several limitations. First, we were unable to assess the actual nitrogen allocation to metabolic processes because of the lack of data on major nitrogen pools within leaves and the entire plant. Second, the use of soil nutrient availability as a categorical variable may not accurately capture the true nutrient availability to plants. Future studies should directly measure multiple metrics of nutrient availability to establish a more precise link between nutrient availability and plant traits.

Additionally, the generalisability of our findings, particularly in relation to ESMs is uncertain. It remains unclear how species-level, climate-driven leaf nitrogen responses to soil nitrogen supply scale to the community level, considering factors like species turnover and assemblage shifts. Further research is needed to assess how local ecological processes influence leaf nitrogen responses to soil nutrient supply in conjunction with climatic factors in grasslands and non-grassy ecosystems.

5 | Conclusions

Our findings reveal the strong influence of leaf nitrogen demand on leaf nitrogen responses to soil nitrogen supply. In drier and colder conditions, leaf nitrogen response was higher than in wetter and warmer conditions, likely to compensate for decreasing water availability (χ) and photosynthetic enzymatic activity, respectively. However, this heightened nitrogen response under high-demand environments appears unaffected by the percentage changes in aboveground biomass. This suggests that quantifying leaf nitrogen demand should be a priority for future development of ESMs.

Author Contributions

A.C., E.F.W., and N.G.S. conceived of the study with input from all co-authors. A.C. and N.G.S. performed the analyses. A.C. drafted the manuscript. All co-authors provided input on subsequent drafts.

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Data Availability Statement

All data and code used for these analyses are available at: https://doi.org/10.5281/zenodo.13952400.

Peer Review

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.