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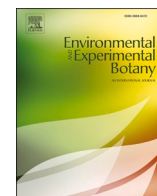
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Research Paper

Low nitrogen supply decreases the mesophyll conductance and limits the effects of elevated CO₂ on photosynthesis in winter wheat across multiple generations

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ABSTRACT

Although the interaction between elevated CO₂ (e[CO₂]) and nitrogen (N) availability on photosynthesis has been extensively studied within single generations, their interplay across multiple generations remains poorly understood. This study investigated the photosynthetic responses of wheat (*Triticum aestivum* L.) to atmospheric CO₂ concentration (400 μmol mol⁻¹ for Control vs. 800 μmol mol⁻¹ for e[CO₂]) and soil N supply (0.5 vs. 1.5 g N pot⁻¹) in plants from the first (F1) and fourth (F4) generations of a lineage exposed to these conditions. We quantitatively assessed the leaf photosynthetic limitations imposed by stomatal conductance (g_s), mesophyll conductance (g_m), and biochemistry (V_{c,max}) during the grain filling stage. Compared to the strong effects of CO₂ concentrations and N levels, changes attributable to generational exposure were minor. Under e[CO₂] and sufficient N, both generations exhibited enhanced chloroplast CO₂ concentration and net photosynthesis rate without declines in g_m. Low N under e[CO₂] significantly reduced g_m (68–73%) and net photosynthesis rate (32–41%), with the magnitude of reduction being similar between F1 and F4 plants, indicating that the immediate N status had a far greater impact than transgenerational exposure. Photosynthesis was primarily limited by biochemistry (57%) under e[CO₂] with adequate N, but shifted to a strong co-limitation by g_m (38%) and biochemistry (44%) under low N, a pattern consistent in both generations. Path analysis confirmed that N availability had the most substantial effect on the net photosynthesis rate (path coefficient = 0.95), whereas generational effects were minimal. Multivariate analysis showed apparent clustering by CO₂ and N treatments, but complete overlap between generational groups. We conclude that the positive effect of e[CO₂] on wheat photosynthesis is strongly influenced by soil N availability within the timeframe of four generations. The photosynthetic apparatus showed high plasticity to current conditions, with limited modification by transgenerational effects. These results highlight that improving nitrogen management is essential for maintaining wheat photosynthetic performance and, by extension, potential yield, in future high-CO₂ environments. This underscores the continued primacy of nitrogen management for securing crop productivity in a changing climate.

1. Introduction

The annual average atmospheric CO₂ concentration has reached 418 μmol mol⁻¹, a substantial increase from the pre-industrial baseline of approximately 280 μmol mol⁻¹ (Masson-Delmotte et al., 2021). In a worst-case scenario, it is predicted to reach 1300 μmol mol⁻¹ (RCP 8.5)

at the end of this century (Masson-Delmotte et al., 2021). Elevated CO₂ concentration (e[CO₂]) stimulates plant growth in some studies, but not in others (Ainsworth and Long, 2020). A key determinant of this variability is soil nitrogen (N) availability, which is a critical modulator of the magnitude and direction of photosynthetic responses in C₃ plants (Gao et al., 2020; Lau et al., 2008). Furthermore, N limitation already

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constrains crop productivity across many agricultural regions—a challenge expected to be exacerbated by rising atmospheric CO₂.

While most knowledge of plant acclimation to e[CO₂] stems from single-generation studies (Alemineu and Abera, 2020; Jahan et al., 2021), plants in natural and agricultural systems will experience rising CO₂ over multiple generations. This long-term exposure necessitates consideration of transgenerational effects, where parental environment shapes offspring phenotype, potentially through mechanisms such as epigenetic modifications, maternal provisioning, and subtle adaptive shifts (Fitz-James and Cavalli, 2022; Herman and Sultan, 2011). Critically, these multi-generational responses may yield fundamentally different acclimation trajectories compared to those observed within a single lifespan (Burton and Greer, 2022).

Emerging evidence confirms that transgenerational effects of e[CO₂] are ecologically and agronomically relevant. For example, maternal e[CO₂] experience can influence offspring performance in grasslands (Lau et al., 2008), and in crops like wheat, multi-generational e[CO₂] exposure has been shown to alter seed metabolism and leaf functional traits (Li et al., 2023, 2024, 2019). In hexaploid wheat, these responses appear to accumulate gradually, with detectable physiological and biochemical changes often emerging after three to four generations of continuous exposure (Li et al., 2019; Wang et al., 2024). This delayed emergence is plausible given the allohexaploid genome (AABBDD) of wheat, which may buffer phenotypic shifts through subgenomic interactions and homeologous gene redundancy (Cheng et al., 2024). Against this background, comparing the first (F1) and fourth (F4) generations provides a practical framework for testing whether prolonged e[CO₂] exposure leads to cumulative adjustments in photosynthetic capacity in hexaploid wheat. However, a critical gap persists in understanding how such multi-generational exposure specifically reprograms the core process of photosynthesis—the ultimate driver of growth and yield. Although some Free-Air CO₂ Enrichment (FACE) studies suggest that key photosynthetic traits respond directionally similarly across timescales (Saban et al., 2019), controlled multigenerational experiments indicate that the magnitude, persistence, and regulation of these responses are more complex and strongly modulated by N availability (Fitz-James and Cavalli, 2022; Wang et al., 2024). Therefore, the interactive effects of e[CO₂] and nitrogen availability across multiple generations likely create a unique physiological context that can be elucidated only through targeted, multi-factorial experiments across plant lineages.

A central process in these dynamics is photosynthetic acclimation. A well-documented pattern is that e[CO₂] initially stimulates the net photosynthetic rate (A_N) in C₃ crops, but this stimulation is often downregulated following prolonged exposure within a generation (Ainsworth and Long, 2020). Mechanisms proposed for this photosynthetic acclimation include reduced Rubisco content due to altered N partitioning (Byeon et al., 2021), suppression of photosynthetic gene expression by carbohydrate accumulation (Wingler and Henriques, 2022), and inhibition of nitrate assimilation (Adavi and Sathee, 2021; Bloom et al., 2020). Furthermore, a decline in mesophyll conductance (g_m), which influences CO₂ diffusion from the intercellular space (C_i) to the site of carboxylation inside the chloroplast stroma (C_c), has been proposed as a mechanism of photosynthetic downregulation in C₃ crop plants (Jahan et al., 2021; Kitao et al., 2015). Historically, g_m was assumed to be infinite or sufficiently large to avoid being rate-limiting, leading to its common omission from estimates of key photosynthetic parameters such as the maximum carboxylation rate ($V_{c,max}$) and the electron transport rate (J_{max}) (Evans, 2021; Flexas et al., 2012). However, a substantial body of evidence has accumulated over the past two decades indicating that g_m is finite and imposes a significant constraint on photosynthesis by reducing C_c relative to C_i (Evans, 2021; Nadal and Flexas, 2019). For instance, g_m was reported to be 0.16–0.39 mol CO₂ m⁻² s⁻¹ bar⁻¹ in bean, 0.20–0.64 mol CO₂ m⁻² s⁻¹ bar⁻¹ in wheat, and 0.3–1.8 mol CO₂ m⁻² s⁻¹ bar⁻¹ in cotton in previous studies (Flexas et al., 2008). In general, annual or biannual herbaceous crops have a g_m around 0.4 mol CO₂ m⁻² s⁻¹ bar⁻¹ (Flexas et al., 2008), which was

similar to the values of stomatal conductance (g_s). Including g_m when calculating photosynthetic parameters, such as $V_{c,max}$ and J_{max} , permits an accurate evaluation of the relative limitations of CO₂ diffusion and carboxylation in photosynthesis. Because $V_{c,max}$ and J_{max} are defined based on the CO₂ at the site of ribulose 1,5-bisphosphate (RuBP) carboxylation, any effect of growth CO₂ on g_m may alter the mechanisms of photosynthetic acclimation to e[CO₂] reported in several studies (Evans, 2021; Flexas et al., 2008; Singaas et al., 2004). Quantifying g_m is therefore essential for accurately assessing the relative limitations of diffusion and biochemistry, and for resolving conflicting reports on acclimation mechanisms (Evans, 2021; Flexas et al., 2008). Despite this, it remains entirely unknown how multi-generational e[CO₂] exposure, especially under varying N supply, reshapes the profile of photosynthetic limitations, including the potential role of g_m .

In this study, we quantified the interactive effects of multigenerational e[CO₂] exposure and N availability on g_m and, consequently, the limitation profile of photosynthesis. We focus on two hypotheses: (i) that the enhancement of photosynthesis by e[CO₂] will be significantly constrained by low soil N levels, and (ii) that this constraint will involve a prominent role of reduced g_m , and that the strength of these effects (of N and g_m) will be evaluated across multiple generations. To test these hypotheses, we employed a multifactorial design to quantify the limitations caused by g_s , g_m , and biochemistry ($V_{c,max}$, J_{max}) in winter wheat under conditions of multigenerational exposure to e[CO₂] and N availability. This approach allows us to move beyond previous single-generation acclimation studies by directly probing whether and how the fundamental bottlenecks to photosynthesis are reconfigured by concurrent and multigenerational factors.

2. Materials and methods

2.1. Experimental design

The multigenerational CO₂ exposure experiments were conducted in two climate-controlled glasshouse cells at the University of Copenhagen's Campus Taastrup (55°40'6.61"N, 12°18'25.62"E), Denmark. The potted plants were grown from germination to harvest with either ambient CO₂ concentrations (a[CO₂]: 400 μmol mol⁻¹) or elevated CO₂ concentrations (e[CO₂]: 800 μmol mol⁻¹). The greenhouse environment was controlled to ensure consistency across generations. Atmospheric CO₂ concentrations in each cell were monitored by a CO₂ Transmitter Series GMT220 (Vaisala Group, Helsinki, Finland) and logged every minute by the climate computer (LCC4, Senmatic, Soendersoe, Denmark). The superstructure comprised acrylic panels with >80% visible light transmittance. Supplemental broad-spectrum LED arrays (model: Philips GreenPower LED) were integrated into the roof framework. An automated system activated the supplemental LEDs, providing a constant intensity of 500 μmol m⁻² s⁻¹, whenever the ambient Photosynthetic Photon Flux Density (PPFD) fell below 300 μmol m⁻² s⁻¹, thus ensuring a consistent 16-hour photoperiod. Air temperature was maintained at 20.5 ± 1.5 °C during the day (06:00–22:00) and 17.0 ± 1.0 °C at night using forced-air heating and cooling systems. Relative humidity was maintained at 65 ± 5% using high-precision humidistats, with misting and dehumidification units. The daily data for the atmospheric CO₂ concentrations, temperature and vapor pressure deficiency (VPD) are shown in the supplementary file (Fig. S1). Each cell measured 8 m × 6 m. Pots from all treatments were arranged in a completely randomized design within the central area of each cell. They were systematically rotated every three to four days to minimize positional effects from potential light or temperature gradients.

A modern hexaploid wheat variety (*Triticum aestivum* L. cv. Jimai 325), released by the Shandong Academy of Agricultural Sciences in China, was used. The experimental lineages were established by successively propagating plants under consistently controlled CO₂ and N conditions across generations. Specifically, F1 seeds harvested from the initial a[CO₂] and e[CO₂] treatments were sown and grown under their

respective, unchanged CO₂ and N levels to produce F2 seeds. This practice of within-treatment propagation was repeated to obtain each subsequent generation. Thus, the F4 seeds used in the main experiment were harvested from parental plants that had undergone three consecutive generations of continuous exposure to their designated atmospheric CO₂ concentration [ambient ($a[\text{CO}_2]$, 400 $\mu\text{mol mol}^{-1}$) or elevated ($e[\text{CO}_2]$, 800 $\mu\text{mol mol}^{-1}$)] and soil N regime [low-N: 0.5 g N pot⁻¹; high-N: 1.5 g N pot⁻¹] (Fig. S2). The fourth generation was selected as the endpoint for assessing multigenerational effects based on our group's sequential research. Earlier studies on F4 plants revealed transgenerational effects on a progressive decline in nutritional quality (e.g., protein, minerals) and substantial reprogramming of N metabolism (Li et al., 2019, 2023). These findings suggest that F4 represents a relevant generational stage at which cumulative physiological and biochemical adjustments to $e[\text{CO}_2]$ may become evident.

According to our earlier experiment, N should be provided at 1.5–2.0 g N per pot for optimum growth of 2 wheat plants per pot (4 L). The 1.5 g N per pot was chosen as an adequate N supply regime. The low-N (0.5 g N) supply regimes were chosen as one-third of the high-N concentrations (unpublished data). The plants received either 1.07 g (0.5 g N) or 3.22 g (1.5 g N) urea (CH₄N₂O) and 3.49 g potassium phosphate monobasic (H₂KO₄P) per pot. Before sowing, all the seeds were treated by hastening germination for 48 h in the dark at 26 °C. The pots (4 L, 21.8 cm in height and 18.7 cm in diameter) were filled with peat (Plugg-och SÅjord, dry matter ca. 110 kg m⁻³, organic matter > 95%, pH = 5.6–6.5, EC = 1.5–2.5 mS cm⁻¹), and two plants were kept in each pot. Two plants per pot were maintained to achieve field-relevant density (74 plants m⁻², within the agronomic range of 200–300 plants m⁻² for winter wheat) while ensuring adequate root space without competition, as verified by pre-trial biomass stability ($\leq 5\%$ variation across 1–3 plants per pot). This design minimized edge effects and preserved physiological representativeness under controlled conditions. Each treatment had 6 pot replicates.

2.2. Gas exchanges

Leaf gas exchange parameters were measured at the stem elongation stage (Z36, the 6th node detectable) and grain filling stage (Z71–Z72, caryopsis water ripe) (Zadoks et al., 1974). The last fully expanded leaves of primary tillers were used to measure gas exchange and chlorophyll fluorescence using an open-flow gas exchange system (GFS-3000FL with a standard measuring head 3010-S and a lamp with fluorometer 3057-FL; Walz, Effeltrich, Germany). Prior to daily measurements, the infrared gas analyzer (IRGA) of the GFS-3000 system was calibrated using a certified calibration gas (400 $\pm$ 2 $\mu\text{mol mol}^{-1}$ CO₂ in N₂ balance; AGA Gas AB, Sweden) and a zero-gas (pure N₂). The flow meter and leaf cuvette temperature sensor were calibrated according to the manufacturer's manual. Instrumental stability was verified daily by measuring a reference leaf or a simulated leaf chamber under standard conditions; variations in A_N and g_s were maintained below 3% during these checks.

Based on our repeated observations from multi-year wheat experiments under contrasting CO₂ concentrations, plants grown under $e[\text{CO}_2]$ reached the flag-leaf measurement stage slightly earlier than those under $a[\text{CO}_2]$. Therefore, plants under $a[\text{CO}_2]$ were measured 3 days later to ensure equivalent physiological leaf age (fully expanded, non-senescent flag leaves) and to minimize ontogenetic confounding effects. All plants were measured between 09:00 and 16:00 in a room with a stable climate. In the leaf cuvette, the CO₂ concentration was set to 400 $\mu\text{mol mol}^{-1}$ for the $a[\text{CO}_2]$ treatment and 800 $\mu\text{mol mol}^{-1}$ for the $e[\text{CO}_2]$ treatment, respectively, using a CO₂ mixture. The cuvette temperature was maintained at 25 °C, PPFD was 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with blue and red light, and leaf-to-air vapor pressure deficit (VPD) was 1.5 kPa. After the leaf reached a steady state, typically after 15 min, the A/PPFD curves were conducted at light intensities of 1500, 1300, 1100, 900, 800, 700, 500, 300, 200, 150, 100, 50, and 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$, with each step

maintained for 3–5 min to reach steady-state conditions. The A/C_i curves were generated by adjusting the cuvette CO₂ concentration to 400, 300, 200, 150, 100, 50, 400, 600, 800, 1000 and 1200 $\mu\text{mol mol}^{-1}$ for ambient CO₂ concentration treatments and adjusting the cuvette CO₂ concentration to 800, 600, 400, 300, 200, 150, 100, 50, 800, 1000 and 1200 $\mu\text{mol mol}^{-1}$ for $e[\text{CO}_2]$ treatments, without removing the leaf from the chamber. To minimize transient responses associated with an immediate change in CO₂ concentration, each A/C_i curve was initiated at the respective growth CO₂ concentration of the treatment. Each CO₂ concentration was maintained for 3–5 min to ensure stable gas exchange before recording.

2.3. Estimation of mesophyll conductance

The g_m was estimated using the variable J method and curve fitting method, which are based on different assumptions and are detailed elsewhere (Sharkey et al., 2007; von Caemmerer, 2000; Warren, 2006). The variable J method is based on the Farquhar-von Caemmerer-Berry (FvCB) model of photosynthesis (Farquhar et al., 1980) and utilizes the relationship between the electron transport rate (J) and A_N (Harley et al., 1992). In the FvCB model, A_N can be predicted using Eq. (1) when the rate of electron transport limits photosynthesis.

$$A_N = \frac{C_c - \Gamma^*}{4C_c + 8\Gamma^*} J - R_L \quad (1)$$

The J was calculated as:

$$J = \Phi_{\text{PSII}} * \text{PPFD} * \alpha \beta \quad (2)$$

Where α is the leaf light absorbance, and β is the fraction of quanta absorbed by PSII. The product $\alpha\beta$ was estimated from the slope of the relationship between $\Phi_{\text{PSII O}_2}$ and $4\Phi_{\text{CO}_2}$ (i.e., the quantum efficiency of gross CO₂ fixation). This was determined by measuring photosynthetic light-response curves under non-photorespiratory conditions, established by precisely controlling the O₂ concentration in the leaf cuvette. Using the GFS-3000 system's integrated gas mixing unit, the O₂ concentration was lowered to 2% by diluting ambient air with pure N₂ from a compressed gas cylinder, while the CO₂ concentration was maintained at 1500 $\mu\text{mol mol}^{-1}$ via the system's CO₂ injector. The actual O₂ concentration was continuously monitored by the system's internal sensor to ensure accuracy (Valentini et al., 1995). The actual photochemical efficiency of photosystem II (Φ_{PSII}) was determined according to Genty et al. (1989) using simultaneous gas exchange and chlorophyll fluorescence measurements. During gas exchange measurements, the leaf cuvette's PPFD was actively maintained at 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ using its integrated LED light source. After the leaf reached a steady state, typically after 15 min, steady-state fluorescence (F_s) was recorded under these actinic light conditions. Subsequently, the maximum fluorescence (F_m') was determined by applying a saturating light pulse of 8000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with a duration of 0.8 s.

$$\Phi_{\text{PSII}} = (F_m' - F_s) / F_m' \quad (3)$$

The CO₂ concentration in the chloroplast stroma (C_c) and g_m was calculated as follows:

$$C_c = \frac{\Gamma^* (J + 8(A_N + R_d))}{J - 4(A_N + R_d)} \quad (4)$$

and

$$g_m = \frac{A_N}{C_i - C_c} \quad (5)$$

Where Γ^* is the CO₂ compensation point in the absence of respiration, A_N is the net photosynthesis, which is taken from gas-exchange measurements under saturating light (PPFD 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at the respective growth CO₂ concentration (400 or 800 $\mu\text{mol mol}^{-1}$), and R_d is the day respiration. For each data point generated, we checked whether

it met the criterion ($10 < dC_c / dA < 50$) (Harley et al., 1992). J is obtained according to Eq. (2).

R_d and Γ^* were determined by the Laisk et al. (1977) method. Briefly, the A/C_i curves were measured over the linear portion of the response curve (at 120, 90, 70, 50, and 40 $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ air}$) under five PPFD levels (40, 100, 160, 220 and 280 $\mu\text{mol m}^{-2} \text{ s}^{-1}$). Then, linear regressions between photosynthetic rate (A_N) and C_i at each level of PPFD were applied for each leaf, and the intersection point of the linear regression was considered as the apparent CO_2 photocompensation point (C_i^* , x-axis) and R_d (y-axis) (von Caemmerer et al., 1994). Γ^* was determined as:

$$\Gamma^* = C_i^* + \frac{R_d}{g_m} \quad (6)$$

The values of $\alpha\beta$, Φ_{PSII} , J , and the independently determined parameters R_d and Γ^* measured in this study are shown in the supplementary data (Table S1). To avoid the potential for large estimation errors associated with low CO_2 concentrations (Harley et al., 1992), g_m values were calculated and reported at an atmospheric CO_2 concentration of 400 $\mu\text{mol mol}^{-1}$, utilizing the R_d and Γ^* parameters derived from the Laisk method (Laisk et al., 1977).

We also estimated g_m utilizing the curve-fitting method introduced by Ethier and Livingston (2010) to validate the g_m values obtained through the variable J method. The core concept of the curve-fitting method is that g_m decreases the curvature of the Rubisco-limited section of an A_N/C_i response curve (Ethier and Livingston 2004). Under conditions limited by Rubisco, the photosynthesis response to CO_2 depends on the kinetic properties of Rubisco (K_c , K_o , C^*) and C_c :

$$A_N = \frac{V_{\text{cmax}}(C_c - \Gamma^*)}{C_c + K_c(O/K_o)} - R_d \quad (7)$$

Where V_{cmax} represents the maximum rate of carboxylation, K_c is the Michaelis-Menten constant for carboxylation, K_o is the Michaelis-Menten constant for oxygenation, and O stands for the oxygen concentration. Substituting C_c with $C_i - A_N/g_m$ results in a quadratic equation. The solution to this equation is the positive root:

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

Where $a = -1/g_m$, $b = [(V_{\text{cmax}} - R_d)/g] + C_i + K_c(1 + O/K_o)$, and

$$c = R_d \left[C_i + K_c \left(1 + \left(\frac{O}{K_o} \right) \right) \right] - V_{\text{cmax}}(C_i - \Gamma^*) \quad (8)$$

Eq. (8) was applied to fit the Rubisco-limited portion of the A/C_i response curves.

We calculated g_m using the curve fitting method and the Excel package developed by Sharkey (2016). The values of K_c and K_o were set according to Sharkey (2016). The Γ^* are provided in Table S1. We graphically identified the point at which the Rubisco-limited portion of the curve transitions to the limitation imposed by RuBP. This was done by plotting the measured A_N against the modeled net photosynthetic rate limited by Rubisco activity (A_c) following the method outlined by von Caemmerer (2000). The g_m estimates obtained from the curve-fitting method are presented in Fig. S3a, and a comparison between the two methods is shown in Fig. S3b.

2.4. Estimation of biochemical parameters involved in photosynthesis

From the A/C_c curves, the maximum carboxylation capacity of Rubisco based on C_c ($V_{\text{cmax Cc}}$) was calculated using the Eq. (11) (Bernacchi et al., 2002):

$$A_c = (C_c - \Gamma^*) / \left(C_c + K_c \left(1 + \left(\frac{O}{K_o} \right) \right) \right) V_{\text{cmax Cc}} - R_d \quad (11)$$

Where the temperature dependence of Rubisco's kinetic parameters

on the C_c basis was estimated following Bernacchi et al. (2002).

The maximum rate of electron transport driving RuBP regeneration based on C_c ($J_{\text{max Cc}}$) was estimated using Eq. (12) (Farquhar et al., 1980; Ethier and Livingston 2004):

$$A_j = \frac{C_c - \Gamma^*}{C_c + 2\Gamma^*} J_{\text{max Cc}} / (4 - R_d) \quad (12)$$

2.5. Estimation of photorespiration rate

Photorespiration rate (F) was estimated from simultaneously measured gas exchange and chlorophyll fluorescence parameters, following the approach of Valentini et al. (1995) and Harley et al. (1992). Under light-saturating conditions, the J is partitioned between the carboxylation and oxygenation reactions of Rubisco. According to the FvCB model, the relationship among J , A_N , R_d , and F can be expressed as:

$$J = 4(A_N + R_d + F) \quad (13)$$

Solving for F yields:

$$F = \frac{J}{4} - (A_N + R_d) \quad (14)$$

Alternatively, the equation can be written as:

$$F = \frac{J - 4(A_N + R_d)}{12} \quad (15)$$

where F is the rate of photorespiratory CO_2 release ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$). The factor 12 arises because 8 electrons are required to reduce one O_2 in the oxygenation reaction, and each photorespiratory CO_2 release corresponds to 2 oxygenations (Valentini et al., 1995).

All parameters used in the calculation were obtained from measurements conducted under the same environmental conditions: saturating photosynthetic photon flux density (PPFD 1500 $\mu\text{mol m}^{-2} \text{ s}^{-1}$) at the respective growth CO_2 concentration (400 $\mu\text{mol mol}^{-1}$ for $a\text{CO}_2$ or 800 $\mu\text{mol mol}^{-1}$ for $e\text{CO}_2$). A_N was taken directly from gas-exchange records at steady state. The electron transport rate J was calculated from chlorophyll fluorescence as described in Eq. (2). R_d was determined by the Laisk method (Laisk et al., 1977) as described earlier.

2.6. Quantitative limitation analyses of photosynthesis

To elucidate the dynamic control of A_N by various functional components, we derived the relative constraints imposed by stomatal conductance (L_s), mesophyll conductance (L_m), and photosynthetic biochemistry (L_b) using the quantitative limitation analysis framework described by Grassi and Magnani (2005).

The core assumption is that A_N is constrained by the sum of fractional limitations ($L_s + L_m + L_b = 1$). Relative changes in A_N are apportioned to parallel relative changes in stomatal conductance to CO_2 (g_{sc}), g_m , and the alternative biochemical capacity ($\partial A_N / \partial C_c$), weighted by their respective control coefficients (relative limitations):

$$\frac{\partial A_N}{A_N} = L_s \frac{\partial g_{sc}}{g_{sc}} + L_m \frac{\partial g_m}{g_m} + L_b \frac{\partial V_{\text{cmax}}}{V_{\text{cmax}}} \frac{\text{max } C_c}{C_c} \quad (16)$$

where ∂g_{sc} , ∂g_m , and $\partial A_N / \partial C_c$ are relative differences between treatments and native limitations were initially calculated as:

$$L_s = \frac{\frac{g_{\text{tot}}}{g_s} \times \frac{\partial A_N}{\partial C_c}}{\frac{g_{\text{tot}}}{g_s} + \frac{\partial A_N}{\partial C_c}} \quad (17)$$

$$L_m = \frac{\frac{g_{\text{tot}}}{g_m} \times \frac{\partial A_N}{\partial C_c}}{\frac{g_{\text{tot}}}{g_m} + \frac{\partial A_N}{\partial C_c}} \quad (18)$$

$$L_b = \frac{g_{\text{tot}}}{g_{\text{tot}} + \frac{\partial A_N}{\partial C_c}} \quad (19)$$

where g_{sc} was derived as $g_s/1.6$, and total conductance (g_{tot}) to CO_2 was calculated as $1/(1/g_{\text{sc}} + 1/g_m)$.

Crucially, we refined the definition of $\partial A_N/\partial C_c$ based on the operating-limitation point determined from the A/C_i curves, using the Bernacchi et al. (2001) enzyme kinetic parameters (K_c , K_o , and Γ^*) adjusted to leaf temperature.

For plants at $a[\text{CO}_2]$ ($400 \mu\text{mol mol}^{-1}$), which operate under Rubisco-limited conditions, $\partial A_N/\partial C_c$ was defined as the partial derivative of A with respect to C_c at the V_{cmax} -limited state (A_c):

$$\partial A_N / \partial C_c = \partial A_N / \partial C_{C_{\text{Rub}}} = \frac{V_{\text{cmax}} (K + \Gamma^*)}{(C_c + K)^2} \quad (20)$$

where $K = K_c (1 + O/K_o)$, and O is the oxygen concentration ($210 \text{ mmol mol}^{-1}$).

For plants at $e[\text{CO}_2]$ ($800 \mu\text{mol mol}^{-1}$), which often shift to RuBP regeneration-limited conditions, $\partial A_N/\partial C_c$ was defined as the partial derivative of A with respect to C_c at the electron transport-limited state (A_j):

$$\partial A_N / \partial C_c = \partial A_N / \partial C_{C_{\text{photo}}} = \frac{J \times 3\Gamma^*}{4(C_c + 2\Gamma^*)^2} \quad (21)$$

This state-dependent calculation provides a more mathematically rigorous representation of biochemical limitations in high- CO_2 environments, encompassing constraints on both Rubisco activity and electron transport rate.

2.7. Leaf nitrogen, biomass and leaf area

For leaf-level trait measurements, five to six flag leaves per treatment were collected for leaf trait analysis. Leaf area was measured with a scanner (LI-COR 3000; Lincoln, NE, USA), after which samples were dried (70°C , 48 h), weighed, and ground. Leaf N concentration was determined by combustion (Flash 2000, Thermo Fisher Scientific, Cambridge, UK), and LMA was calculated accordingly. Photosynthetic nitrogen use efficiency (PNUE) was calculated as the ratio of A_N to leaf N content on an area basis ($\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ N s}^{-1}$), where leaf N content on an area basis was derived from leaf N concentration (g kg^{-1}) and LMA (g m^{-2}). To assess plant growth, single-tiller biomass and tiller number per plant were recorded on the same plants to evaluate growth responses.

2.8. Statistical analysis

A multivariate analysis of variance (MANOVA) was performed to compare the individual effects of CO_2 treatment, N treatment, and generation on photosynthetic characteristics in flag leaves using the R statistical software (version 4.0.0) (R Team, 2022). Mean $\pm$ standard error labeled with different letters indicates a significant difference between the treatments ($P < 0.05$) according to Duncan's multiple range test. Principal component analysis (PCA) was applied to evaluate variation in photosynthetic characteristics across different CO_2 concentrations, soil N supplies, and generations using the FactoMineR package in R. Path analysis was conducted using a maximum-likelihood approach to examine the relationships among growth conditions (CO_2 concentration, N supply, and generation), g_s , g_m , C_c , V_{cmax} , J_{max} , and A_N . The standardized path coefficient indicates the direction and strength of the linear relationship between the variables (Table S3). We constructed the maximum likelihood multiple model using SPSS 20 (<https://www.ibm.com/products/spss-statistics>). We evaluated model fit using the following criteria: the ratio of the chi-square statistic to the degrees of freedom (χ^2/df), the goodness-of-fit index (GFI), the comparative fit index (CFI), and the normed fit index (NFI). The final model showed acceptable fit indices ($\chi^2/\text{df} = 1.94$, GFI = 0.93, CFI = 0.98, NFI = 0.98), supporting the validity of the proposed pathways.

3. Results

3.1. Leaf and plant growth traits

Low soil N supply significantly decreased flag leaf N content ($P < 0.001$), leaf area ($P < 0.001$), leaf biomass ($P < 0.01$), single tiller biomass ($P < 0.01$), and tiller number per plant ($P < 0.001$) (Table 1). Elevated CO_2 significantly increased leaf biomass ($P < 0.01$), PNUE ($P < 0.001$), and leaf mass per area (LMA) ($P < 0.01$), while neither N availability, generational exposure, nor their interactions significantly affected LMA ($P > 0.05$).

Under $e[\text{CO}_2]$, N limitation significantly decreased flag leaf area by 24% and single tiller biomass by 43% in F4 plants, surpassing the changes observed in F1 counterparts. PNUE was consistently higher under $e[\text{CO}_2]$ than under $a[\text{CO}_2]$, with the $\text{CO}_2 \times \text{N}$ interaction being significant ($P = 0.04$). No significant generational effects were detected for PNUE ($P = 0.49$) or for most other growth traits, except for leaf N content and tiller number per plant.

3.2. Leaf gas exchange traits

Air CO_2 concentration, N supply, and generation had significant effects on A_N during both the grain filling and stem elongation stages (Fig. 1). Additionally, air CO_2 concentration and generation showed an interaction effect at the elongation stage ($P < 0.05$). Furthermore, $e[\text{CO}_2]$ significantly increased A_N by 33%–55% during grain filling compared to $a[\text{CO}_2]$, but it did not significantly increase A_N at the stem elongation stage, except in F1 plants under high N conditions. Thus, $e[\text{CO}_2]$ has a greater impact on A_N during the grain filling stage than during the stem elongation stage.

The parameter g_m was assessed through two different methodologies: the variable J method (Fig. 2) and the curve-fitting method (Fig. S3a). There was a statistically significant positive correlation between the g_m estimates derived from both methods ($P < 0.0001$, $R^2 = 0.67$) (Fig. S3b). Therefore, all following analyses were conducted using g_m estimates derived from the variable J method.

According to the results from the variable J method, $e[\text{CO}_2]$ had significant effects on C_i ($P < 0.001$), C_c ($P < 0.001$), and g_s ($P < 0.01$), but did not affect g_m ($P > 0.05$) during the grain filling stage (Fig. 2). The supply of N and the interaction between N and CO_2 concentration significantly influenced C_c and g_m . Specifically, low N supply did not alter C_i , C_c , g_s , and g_m under $a[\text{CO}_2]$; however, it reduced g_m in both F1 (73%) and F4 (68%) plants, and decreased C_c in F1 (36%) plants under $e[\text{CO}_2]$. Generation had significant effects on g_m ($P < 0.05$) and C_i ($P < 0.05$); the interaction of generation and CO_2 did not influence these two parameters.

3.3. Photosynthesis biochemistry parameters and photorespiration rate

The maximum rates of Rubisco carboxylation (V_{cmax}) and the maximum rate of electron transport (J_{max}) were calculated based on C_c (Fig. 3). Both N and the interaction of N and CO_2 significantly affected J_{max} ($P < 0.001$) and V_{cmax} ($P < 0.001$). CO_2 concentration did not have a significant effect on J_{max} and V_{cmax} . F was significantly influenced by CO_2 concentration ($P < 0.001$), N supply ($P < 0.01$), and their interaction ($P < 0.01$). Across both generations, $e[\text{CO}_2]$ substantially reduced F compared to $a[\text{CO}_2]$, with reductions of 80% under high N and 43% under low N ($P < 0.01$). No consistent generational differences in F were observed between F1 and F4 plants.

Moreover, g_m was positively correlated with g_s , C_c and A_N under $e[\text{CO}_2]$, indicating that higher g_m could help to increase C_c and A_N in plants supplied with high N; whereas, lower g_m caused lower C_c and A_N in plants with low N supply (Fig. S5). Under $a[\text{CO}_2]$, g_m was positively correlated with A_N and V_{cmax} , but not with C_c , indicating increased CO_2 accumulation in chloroplasts under low soil N supply.

Table 1

Flag leaf area, biomass, biomass per area (LMA), and nitrogen content, single tiller biomass and tiller number per plant in wheat under two CO₂ concentrations (400 μmol mol⁻¹ and 800 μmol mol⁻¹) in the grain filling stage (Zadoks stage 75, the medium milk stage).

CO ₂	N levels	Generations	Leaf area (cm ²)	Leaf biomass (g)	LMA (g m ⁻²)	Leaf N Content (g kg ⁻¹)	PNUE [μmol (g N) ⁻¹ s ⁻¹]	Tiller Biomass (g)	Tiller number
aCO ₂	Low N	F1	10.66 ± 0.33 ^{abc}	0.13 ± 0.00 ^{ab}	123.57 ± 5.11 ^b	26.26 ± 0.82 ^d	57.41 ± 6.54 ^{bc}	1.45 ± 0.19 ^d	7.8 ± 1.2 ^{bc}
		F4	10.37 ± 0.79 ^{abc}	0.13 ± 0.02 ^b	122.99 ± 10.36 ^b	23.62 ± 0.44 ^{de}	50.15 ± 7.94 ^c	1.44 ± 0.24 ^d	10.5 ± 0.8 ^{abc}
	High N	F1	11.01 ± 1.15 ^{abc}	0.14 ± 0.01 ^{ab}	130.88 ± 12.14 ^{ab}	40.75 ± 0.76 ^a	49.60 ± 5.37 ^c	2.54 ± 0.38 ^{ab}	10.9 ± 1.6 ^{ab}
		F4	11.37 ± 1.31 ^{abc}	0.15 ± 0.01 ^{ab}	130.74 ± 13.18 ^{ab}	37.11 ± 1.19 ^b	52.84 ± 4.68 ^c	2.35 ± 0.44 ^{bc}	10.5 ± 0.8 ^{abc}
eCO ₂	Low N	F1	9.63 ± 0.80 ^{bc}	0.16 ± 0.01 ^{ab}	170.40 ± 8.61 ^a	23.31 ± 0.75 ^e	72.53 ± 6.79 ^{ab}	2.05 ± 0.28 ^c	7.6 ± 1.9 ^c
		F4	9.54 ± 0.19 ^c	0.13 ± 0.03 ^{ab}	139.83 ± 29.27 ^{ab}	21.72 ± 1.16 ^e	77.10 ± 7.24 ^{ab}	1.61 ± 0.23 ^{cd}	9.0 ± 1.5 ^{abc}
	High N	F1	11.86 ± 0.23 ^{ab}	0.17 ± 0.01 ^a	145.06 ± 6.82 ^{ab}	38.22 ± 0.96 ^{ab}	69.32 ± 5.43 ^b	3.04 ± 0.53 ^a	12.4 ± 1.7 ^{ab}
		F4	12.55 ± 0.79 ^a	0.17 ± 0.02 ^{ab}	133.35 ± 7.35 ^{ab}	31.00 ± 0.33 ^c	82.88 ± 6.96 ^a	2.80 ± 0.78 ^{ab}	12.3 ± 2.2 ^a
P values	CO ₂		0.90 ^{ns}	0.004 ^{**}	0.005 ^{**}	0.0004 ^{***}	0.001 ^{***}	0.0002 ^{***}	0.73 ^{ns}
	N		0.0001 ^{***}	0.009 ^{**}	0.51 ^{ns}	0.00001 ^{***}	0.009 ^{**}	0.003 ^{**}	0.0001 ^{***}
	Generation		0.62 ^{ns}	0.19 ^{ns}	0.10 ^{ns}	0.0001 ^{***}	0.49 ^{ns}	0.35 ^{ns}	0.03 [*]
	CO ₂ × N		0.009 ^{**}	0.65 ^{ns}	0.08 ^{ns}	0.03 [*]	0.04 [*]	0.47 ^{ns}	0.10 ^{ns}
	CO ₂ × Generation		0.68 ^{ns}	0.18 ^{ns}	0.11 ^{ns}	0.14 ^{ns}	0.43 ^{ns}	0.31 ^{ns}	0.57 ^{ns}
	N × Generation		0.30 ^{ns}	0.19 ^{ns}	0.45 ^{ns}	0.001 ^{***}	0.57 ^{ns}	0.26 ^{ns}	0.07 ^{ns}
	CO ₂ × N × Generation		0.93 ^{ns}	0.49 ^{ns}	0.47 ^{ns}	0.01 [*]	0.79 ^{ns}	0.52 ^{ns}	0.22 ^{ns}

Data are mean values ± SD, n = 3. Different lowercase letters are significantly different ($P \leq 0.05$) according to Duncan's multiple range test. MANOVA was used to test for the effects of CO₂ concentration, N supply, generation and their interaction on these parameters. *, ** and *** indicate significant levels at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively. 'ns' denotes non-significant differences.

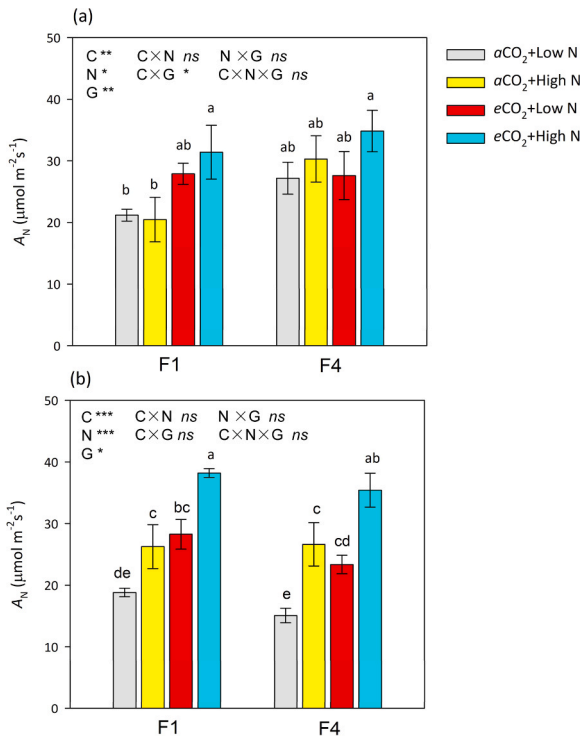


Fig. 1. A_N in wheat plants at the stem elongation stage (a) and the grain filling stage (b) under two atmospheric CO₂ concentrations (400 μmol mol⁻¹ and 800 μmol mol⁻¹). The A_N were measured under saturating light (PPFD of 1500 μmol m⁻² s⁻¹). Data represent mean values ± SD, n = 4–5. Different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test under ambient and elevated CO₂ concentration. C, N and G indicate CO₂ concentration, nitrogen level and generation, respectively; *, ** and *** indicate significant levels at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively; 'ns' denotes non-significant differences.

3.4. Relative limitation analyses on photosynthesis

Both N and the N-CO₂ interaction had significant effects on the relative mesophyll limitation (L_m) and the biochemical limitation (L_b) (Table 2). Under e[CO₂], the L_b (55%–59%) primarily limited photosynthesis under adequate N supply, whereas both L_m (35%–40%) and L_b

(42%–46%) jointly restricted photosynthesis under low N conditions (Fig. 4). Moreover, low N supply increased L_m by 84% for F1 and 82% for F4 plants under e[CO₂], both of which were more than that under a [CO₂]. The relative stomatal limitation (L_s) was not affected by the CO₂, N and their interaction (Table 2). Generation did not have significant effect on L_s , L_m and L_b .

In relation to plants grown under a[CO₂], e[CO₂] did not affect g_s and g_m under high N supply (Fig. 5a, c). Under low N conditions, e[CO₂] reduced g_s in both F1 (–52%) and F4 (–26%) plants, and decreased g_m (–32%) in F4 plants. Meanwhile, e[CO₂] increased L_s in F1 plants (42%) under low N (Fig. 5b), whereas it decreased L_m for F1 plants (18%; Fig. 5d). The e[CO₂] decreased $V_{c,max}$ C_c and increased L_b under high N, while increased $V_{c,max}$ C_c (except F1 plants) and did not change L_b under low N (Fig. 5e, f).

3.5. Path and multivariate analysis of photosynthetic characteristics

Principal component analysis (PCA) was employed to visualize the overall patterns of photosynthetic traits in response to CO₂ concentration, N supply, and generational exposure (Fig. 6). PC1 and PC2 explained 44.3% and 23.8% of the total variation, respectively. The growth CO₂ concentration clearly separated the data into two distinct clusters (Fig. 6a): the a[CO₂] cluster aligned with vectors for g_s , $V_{c,max}$ C_c, J_{max} C_c, and L_m , whereas the e[CO₂] cluster aligned with C_i , C_c , and A_N . This indicates that enhanced A_N under e[CO₂] was primarily driven by increased C_c rather than by changes in biochemical capacity ($V_{c,max}$ C_c, J_{max} C_c). Nitrogen supply also separated the data into two clusters (Fig. 6b): high N conditions were associated with A_N , g_s , g_m , $V_{c,max}$ C_c, and J_{max} C_c, while low N conditions were associated with increased L_m . In contrast, the F1 and F4 generations showed nearly complete overlap (Fig. 6c), indicating minimal transgenerational effects on overall photosynthetic performance relative to the dominant influences of CO₂ and N treatments.

To further quantify the causal relationships underlying these patterns, we employed maximum likelihood path analysis (Fig. 7, Table S3). The model confirmed that N supply exerted the strongest positive effect on A_N (path coefficient = 0.95, $P < 0.01$), primarily mediated through g_m (0.82, $P < 0.01$), C_c (1.0, $P < 0.01$), $V_{c,max}$ C_c (0.49, $P < 0.01$), and J_{max} C_c (0.37, $P < 0.05$). Elevated CO₂ also significantly enhanced A_N (0.70, $P < 0.01$), but this effect operated mainly through increased C_c (0.95, $P < 0.01$) and C_i (0.58, $P < 0.01$) rather than through g_m or biochemical parameters. Generation had a minor but significant negative effect on g_m (–0.25, $P < 0.05$), yet it did not

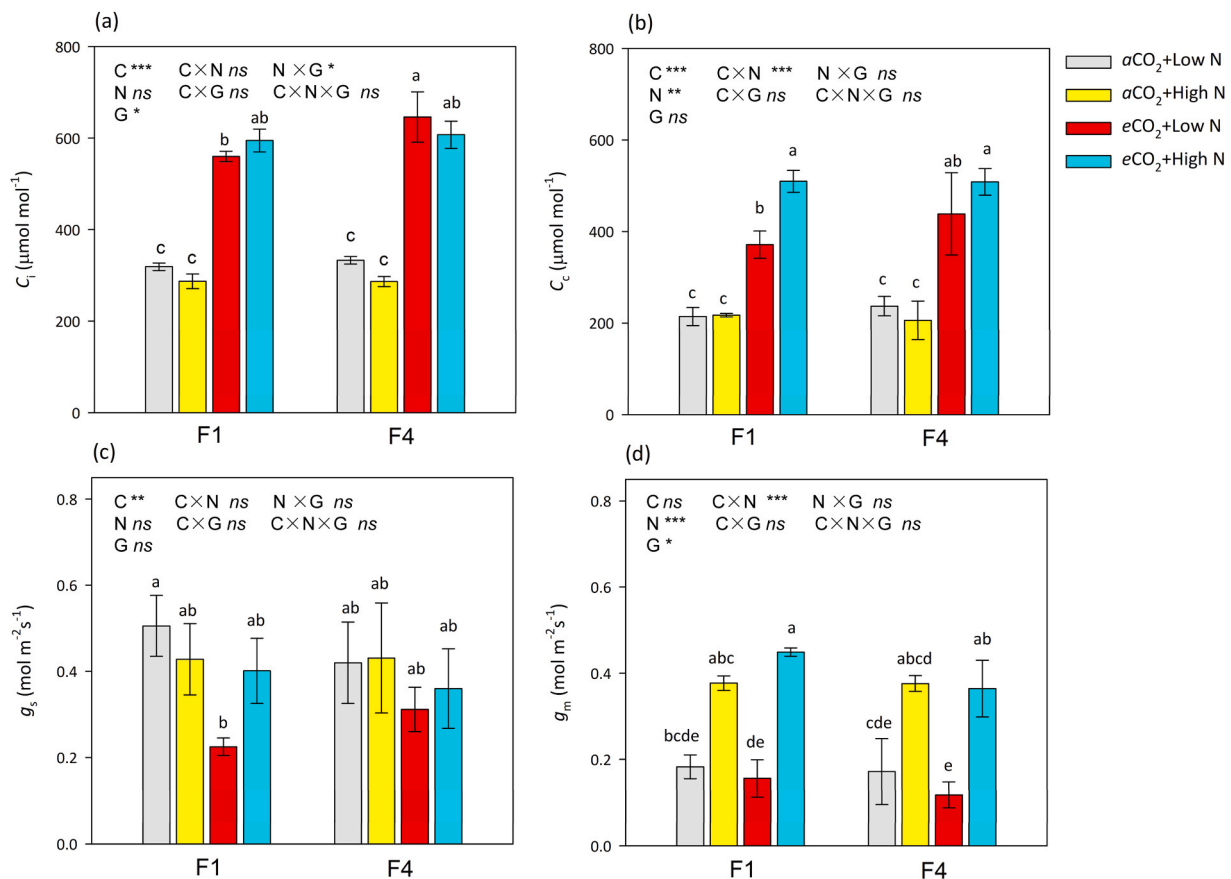


Fig. 2. Intercellular CO₂ concentration (C_i , a), Chloroplast CO₂ concentration (C_c , b), stomatal conductance (g_s , c), and mesophyll conductance (g_m , d) in flag leaves of wheat plants during the grain filling stage. Plants were grown under two atmospheric CO₂ concentrations: ambient (400 $\mu\text{mol mol}^{-1}$) and elevated (800 $\mu\text{mol mol}^{-1}$). The g_s and C_i were measured under saturating light (PPFD of 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$); g_m and C_c were estimated using the variable J method. Data represent mean values $\pm$ SD, $n = 3$. Different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test under ambient and elevated CO₂ concentration. C, N and G indicate CO₂ concentration, nitrogen level and generation, respectively; *, ** and *** indicate significant levels at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively; 'ns' denotes non-significant differences.

significantly influence C_i , C_c , $V_{c,\max}$, C_c , $J_{\max}$, C_c , or A_N . Together, these analyses demonstrate that immediate N availability and CO₂ concentration overwhelmingly govern photosynthetic performance, with any transgenerational effects being biologically negligible within the four-generation timeframe examined.

4. Discussion

This study elucidates the complex interaction among atmospheric CO₂ levels, soil N availability, and generational exposure in winter wheat photosynthesis. Our findings demonstrate that while $e[\text{CO}_2]$ enhanced A_N under adequate N supply, its efficacy was critically dependent on concurrent soil N fertility. Low N availability not only diminished the A_N response but also fundamentally reconfigured the limiting factors of photosynthesis under $e[\text{CO}_2]$, shifting the primary limitation from biochemical to a co-limitation by mesophyll diffusion and biochemistry. Critically, over four generations, photosynthetic traits exhibited high responsiveness to immediate conditions, with any changes attributable to generational exposure being minor in both statistical effect size and biological magnitude compared to the overwhelming influence of immediate CO₂ and N conditions. This emphasizes the primacy of contemporary resource availability in shaping photosynthetic performance within the studied timeframe.

4.1. Generational stability of photosynthetic responses and the overriding influence of N availability

Our core finding is the remarkable stability of the photosynthetic response profile across generations, which was predominantly governed by current N availability. Under $e[\text{CO}_2]$ with high N, key parameters like g_m remained stable from F1 to F4. This contrasts with reports in species like *Arabidopsis* (Mizokami et al., 2019) and rice (Yang et al., 2021), where $e[\text{CO}_2]$ induced anatomical changes (e.g., cell wall thickening, reduced chloroplast exposure) that suppressed g_m . This observed constitutive stability in wheat may be partly attributed to its genetic background as a modern cultivar selected for high performance under optimal conditions (Cheng et al., 2024). We postulate that sufficient N supports the synthesis and function of proteins essential for internal CO₂ diffusion, including aquaporins (PIPs) that facilitate transmembrane CO₂ transport and carbonic anhydrase (CA), which enhances CO₂ dissolution and its conversion to HCO_3^- in the stroma (Evans, 2021; Flexas et al., 2012). Additionally, adequate N may have maintained mesophyll cell wall elasticity and thickness within a range that did not significantly increase diffusive resistance, while also maintaining chloroplast distribution and alignment along the cell periphery, thus maximizing surface area for CO₂ uptake (Byeon et al., 2021; Zhu et al., 2024). Thus, the observed stability is best explained by an N-enabled maintenance of the mesophyll's structural and functional integrity. This stability in g_m was consistent across generations and was essential for sustaining enhanced C_c (134–147%) and A_N (33–55%) under $e[\text{CO}_2]$ with high N supply.

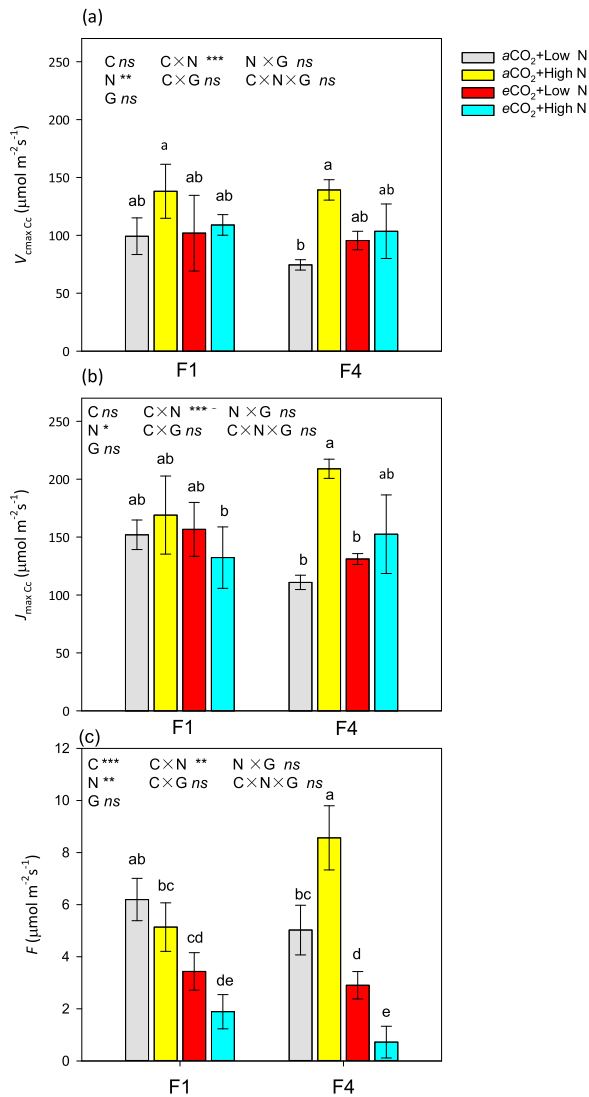


Fig. 3. The maximum rate of rubisco carboxylation based on C_c ($V_{c,max Cc}$, a), the maximum rate of electron transport based on C_c ($J_{max Cc}$, b), and the photorespiration rate (F , c) in flag leaves of wheat plants grown at two atmospheric CO₂ (400 $\mu\text{mol mol}^{-1}$ and 800 $\mu\text{mol mol}^{-1}$) in the grain filling stage. Data represent mean values $\pm$ SD, $n = 3$. Different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test under ambient and elevated CO₂ concentration. C, N and G indicate CO₂ concentration, nitrogen level and generation, respectively; *, ** and *** indicate significant levels at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively; 'ns' denotes non-significant differences.

Conversely, low N supply under $e[\text{CO}_2]$ significantly reduced g_m (by 68–73%) and A_N , with the magnitude of reduction being similar between the F1 and F4 generations. This underscores the high sensitivity of photosynthesis to immediate N limitation. The lack of a progressive change across generations suggests that the photosynthetic apparatus did not acclimate in a way that substantially mitigated or exacerbated the low-N stress over the studied generational timeframe. Since N constitutes a fundamental element of amino acids and nucleotides, its deficiency is likely to inhibit the synthesis and turnover of essential proteins that regulate g_m , such as PIP aquaporins and CA enzymes (Evans, 2021; Flexas et al., 2012). Consequently, this diminishes facilitated CO₂ transport across membranes and through the aqueous phase. Furthermore, N deficiency commonly induces anatomical alterations such as increased mesophyll cell wall thickness and reduced chloroplast surface area exposed to intercellular airspace, which together increase

Table 2

Summary of the multivariate analysis of variance (MANOVA) to test for the effects of CO₂ concentration, N supply, generation and their interaction on relative stomatal (L_s), mesophyll (L_m), and biochemical limitations (L_b) to photosynthesis.

	P values		
	L_s	L_m	L_b
CO ₂	0.90 ^{ns}	0.02*	0.01**
N	0.04*	0.001***	0.02*
Generation	0.41 ^{ns}	0.54 ^{ns}	0.91 ^{ns}
CO ₂ $\times$ N	0.16 ^{ns}	0.53 ^{ns}	0.007**
CO ₂ $\times$ Generation	0.21 ^{ns}	0.86 ^{ns}	0.70 ^{ns}
N $\times$ Generation	0.65 ^{ns}	0.88 ^{ns}	0.31 ^{ns}
CO ₂ $\times$ N $\times$ Generation	0.26 ^{ns}	0.40 ^{ns}	0.56 ^{ns}

*, ** and *** indicate significant levels at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively. 'ns' denotes non-significant differences. The measurements were repeated 3 times (leaves) per treatment.

diffusive resistance (Xiong et al., 2015; Zhu et al., 2024). The accumulation of non-structural carbohydrates under $e[\text{CO}_2]$ could exacerbate these structural modifications under low N (Li et al., 2023b). Importantly, under $e[\text{CO}_2]$ combined with low N supply, the suppression of g_m was more pronounced than that of g_s or $V_{c,max Cc}$, indicating a particular sensitivity of the mesophyll diffusion pathway to N deprivation in future high-CO₂ environments. This reduction in CO₂ availability at the chloroplast, driven by compromised g_m under low N, also profoundly impacted F . While $e[\text{CO}_2]$ typically suppresses F by elevating C_c , the significant decrease in C_c observed under low N (even with $e[\text{CO}_2]$) led to a marked increase in F ($P < 0.01$). This demonstrates that low N can partially negate the photorespiratory benefits of $e[\text{CO}_2]$ by lowering the CO₂/O₂ ratio at the site of carboxylation, thereby shifting Rubisco activity towards oxygenation. Consistent with other traits, F showed no generational differences between F1 and F4 (Fig. 3), thus underscoring that the response of photorespiration to $e[\text{CO}_2]$ is not a consequence of transgenerational acclimation.

It is worth noting that the low N treatment in this study (0.5 g N pot⁻¹) induced moderate rather than severe N deficiency. Leaf N contents in low N plants ranged from 21.7 to 26.2 g kg⁻¹ (Table 1), which is above the critical deficiency threshold for wheat at grain filling (Justes et al., 1994). This explains the sustained $V_{c,max Cc}$ under low N (Fig. 3) and indicates that our findings reflect a moderate N limitation rather than an extreme stress condition, which is relevant to many rainfed agricultural systems.

4.2. Reassessment of photosynthetic limitations under future climate scenarios

The quantitative limitation analysis, based on g_m and C_c data, reveals a stable reorganization of photosynthetic limitations by N availability (Xie et al., 2020; Zeng et al., 2023). In this study, "diffusional limitations" are defined as the sum of gas-phase diffusion barriers through the stomata (L_s) and the finite mesophyll conductance (L_m) barriers to CO₂ transfer, as distinguished from the broader biochemical and photochemical potential within the mesophyll (Grassi and Magnani, 2005; Jones, 1985). Under $e[\text{CO}_2]$ with high N, photosynthesis was primarily biochemically limited, consistent with the classical view of CO₂ saturation (Croce et al., 2024; Suzuki et al., 2021). In contrast, under $e[\text{CO}_2]$ with low N, a stable co-limitation pattern emerged between mesophyll conductance and biochemical capacity, which was consistent across the F1 and F4 generations. This stable co-limitation state has significant implications: in persistently N-limited environments, improving only a single limiting factor (e.g., enhancing g_m) may yield limited benefits, as the other factor would promptly become the primary constraint. Consequently, our findings advocate for integrated adaptation strategies that concurrently address diffusional and biochemical limitations or, more effectively, enhance nitrogen use efficiency (NUE) to mitigate the

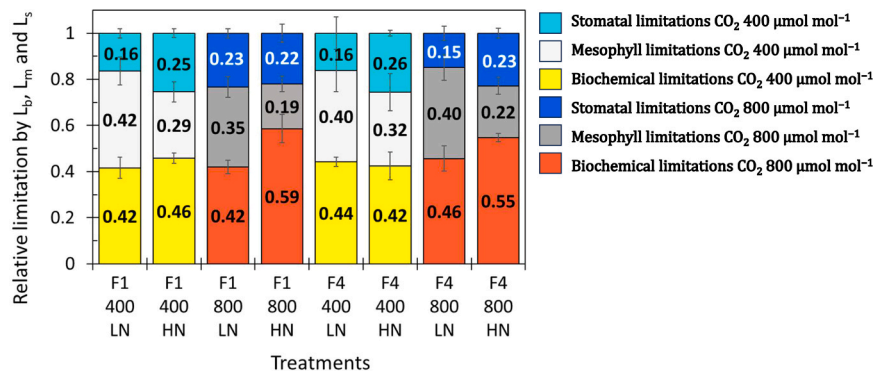


Fig. 4. The relative contributions of stomatal (L_s), mesophyll conductance (L_m), and biochemical limitations (L_b) to photosynthetic constraints are quantified under ambient ($a[CO_2]$, $400\ \mu mol\ mol^{-1}$; L_s : light blue, L_m : light gray, L_b : yellow) and elevated CO_2 ($e[CO_2]$, $800\ \mu mol\ mol^{-1}$; L_s : dark blue, L_m : dark gray, L_b : red) during grain filling. Values represent means $\pm$ SD ($n = 3$ biological replicates), with lowercase letters denoting statistically significant differences among treatments ($P < 0.05$) as detailed in Table 2.

root cause of N limitation in future high- CO_2 climates.

4.3. The primacy of contemporary N availability over transgenerational effects

Our data robustly demonstrate that immediate N availability is the dominant factor controlling photosynthetic performance under $e[CO_2]$. Although statistically significant generational differences were detected for a few parameters (e.g., g_m , C_i), their practical or biological significance was limited. This is evidenced by: 1) the complete overlap of generational clusters in the PCA (Fig. 6c); 2) the negligible path coefficient for generation (-0.25) compared to that for N availability (0.95 , $P < 0.01$); and 3) the direct comparison of effect sizes—for instance, the difference in A_N between high- and low-N treatments under $e[CO_2]$ ($\sim 40\%$) was an order of magnitude greater than any generational difference observed. Consistent with this pattern, PNUE responded primarily to the $CO_2 \times N$ interaction ($P = 0.04$), with no generational difference ($P = 0.49$), further reinforcing that even nitrogen use efficiency is governed by immediate resource availability rather than transgenerational history (Fig. 3). This suggests that the photosynthetic response framework of winter wheat is highly plastic and predominantly governed by current N status, with no evidence of strong transgenerational programming of photosynthetic capacity across four generations in this system.

The relatively subtle transgenerational signals observed in wheat contrast with findings in diploid species like rice, where multigenerational $e[CO_2]$ exposure significantly reduced photosynthetic benefits by the fifth generation (Lv et al., 2022). This discrepancy likely reflects the unique evolutionary and genetic architecture of hexaploid wheat (*Triticum aestivum* L., AABBDD). We propose that the allohexaploid genome provides a robust "genetic buffering" mechanism that stabilizes complex traits like photosynthesis across generations. Due to its three homoeologous subgenomes, wheat possesses extensive functional redundancy; homoeologous gene copies can compensate for heritable epigenetic modifications or regulatory shifts occurring in a single subgenome, thereby maintaining a stable "consensus" transcriptomic and proteomic output (Cheng et al., 2024; Dubcovsky and Dvorak, 2007).

This genomic buffering likely underpins the high phenotypic plasticity characteristic of modern wheat cultivars. Such plasticity allows for efficient, within-generation physiological rebalancing—for instance, through rapid N reallocation—thereby enabling the plant to absorb environmental fluctuations like rising CO_2 without the immediate need for permanent transgenerational adjustments (Bellasio, 2025; Mao et al., 2023). In this context, the stability of g_m and other photosynthetic traits across F1–F4 generations may not signify a lack of response, but rather a genome-stabilized resilience that favors flexible individual acclimation over fixed intergenerational shifts. While this hypothesis explains our

physiological observations, verifying the precise role of subgenomic interactions in transgenerational stability will require integrated "omics" analyses across even longer evolutionary timescales.

4.4. Limitations and future perspectives

This study provides a focused mechanistic understanding of photosynthetic acclimation in winter wheat across four generations under controlled $e[CO_2]$ and N regimes. The findings robustly establish the primacy of contemporary N management within this framework. To provide context for these conclusions and inform future research, it is important to consider several factors and potential extensions.

Firstly, our findings, based on a single high-yielding cultivar ('Jimai 325') under controlled conditions, should be validated across diverse germplasm and in dynamic field environments (e.g. FACE facilities). Future screening should prioritize traits central to photosynthetic resilience under elevated CO_2 and low N, particularly g_m , N use efficiency, and related aspects of N allocation and source–sink coordination. Coupling such phenotyping with genome-wide association approaches could help identify loci associated with superior performance and adaptive capacity. These efforts would clarify whether the observed g_m stability and N-dominant regulation are consistent across broader genetic backgrounds and under interacting biotic and abiotic stresses.

Secondly, we acknowledge the inherent uncertainties in gas-exchange-based g_m estimations, which rely on non-verifiable assumptions regarding the stability of Γ^* , R_d , and stomatal uniformity (Pons et al., 2009). Although the high consistency between our two independent estimation methods provides confidence in the recorded trends, physiological data alone cannot fully resolve the underlying mechanisms. Specifically, the absence of epigenetic or transcriptomic screening in this study remains a limitation, as such molecular profiling could help determine whether CO_2 - or N-induced transgenerational "memory" is mediated by specific DNA methylation patterns or changes in the expression of regulatory gene networks. The transgenerational stability of g_m and its sensitivity to N likely involve a complex interplay between upstream regulatory signals (e.g., genetic or epigenetic N-signaling) and downstream physical manifestations.

Finally, future research integrating molecular-genetic tools with quantitative leaf anatomy is essential. Specifically, direct measurements of anatomical traits (e.g., cell wall thickness and chloroplast surface area) and biochemical drivers (e.g., aquaporin and carbonic anhydrase activity) are needed, ideally coupled with transcriptomic analysis, to provide a holistic view of the structural and molecular constraints on g_m over longer evolutionary timescales (e.g., >10 generations).

In summary, this study demonstrates that immediate N availability, not transgenerational effects, governs photosynthetic performance in wheat over four generations. These findings simplify predictive

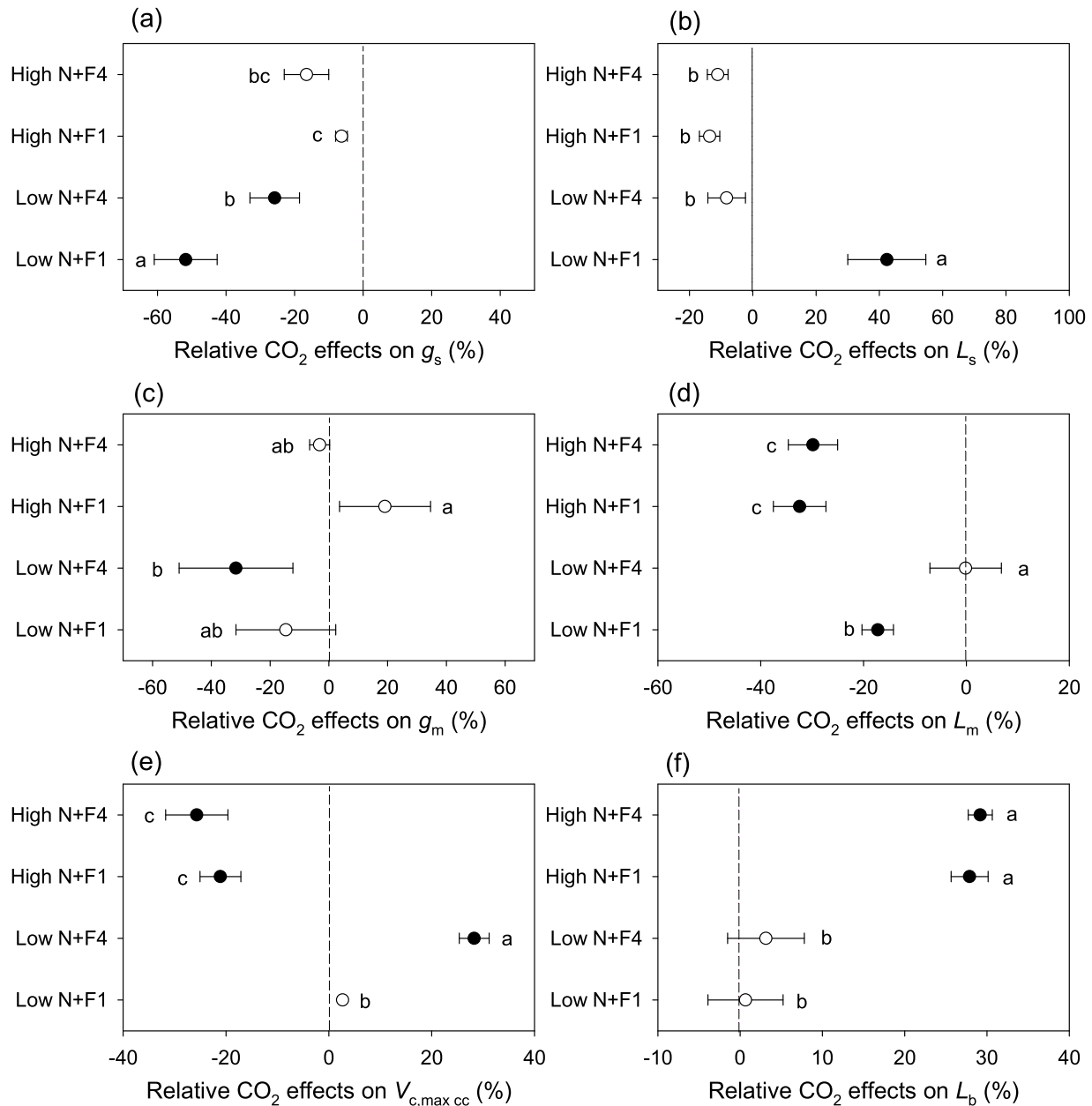


Fig. 5. The relative CO₂ effects on stomatal conductance (a), stomatal limitation (b), mesophyll conductance (c), mesophyll limitation (d), the maximum rate of rubisco carboxylation based on both C_c (e), and biochemistry limitation (f) in the grain filling stage. Values of relative CO₂ effects were calculated by $(\text{Values}_{e\text{CO}_2} - \text{Values}_{a\text{CO}_2}) / \text{Values}_{a\text{CO}_2} \times 100\%$. Open cycles represent the non-significant influence by $e[\text{CO}_2]$ exposure, and black cycles represent that the significant influence by $e[\text{CO}_2]$ exposure. Different small letters mean significant difference between treatments.

modeling and underscore that sustainable N management remains the primary lever for maintaining productivity under future CO₂-enriched climates, while providing a rigorous physiological foundation for subsequent mechanistic investigations.

5. Conclusion

This study highlights the main factors driving photosynthetic acclimation in winter wheat subjected to multigenerational $e[\text{CO}_2]$. We demonstrate that the benefits of $e[\text{CO}_2]$ on A_N are critically dependent on concurrent soil N availability, whereas transgenerational effects have a quantitatively negligible influence relative to this overriding factor. Under sufficient N supply, $e[\text{CO}_2]$ enhanced A_N across generations without suppressing g_m . In contrast, N limitation under $e[\text{CO}_2]$ substantially reduced both A_N and g_m , triggering a fundamental shift in the

primary photosynthetic limitation from biochemical capacity alone to a stable, persistent co-limitation by both mesophyll conductance and biochemical capacity. Although minor generational variations in some traits were detected, their scale was dwarfed by the instantaneous effects of N supply.

Collectively, these findings underscore that the near-term resilience and photosynthetic performance of wheat in a high-CO₂ climate will be determined primarily by sustainable N management strategies, within the genetic potential of current cultivars, rather than by adaptive processes unfolding over a few generations. Our results elucidate a physiological framework in which contemporary resource availability is the principal driver of photosynthetic plasticity. From an applied perspective, this underscores the urgent need to enhance NUE in cropping systems. Future efforts should therefore prioritize integrated strategies that combine improved N management with the development of wheat

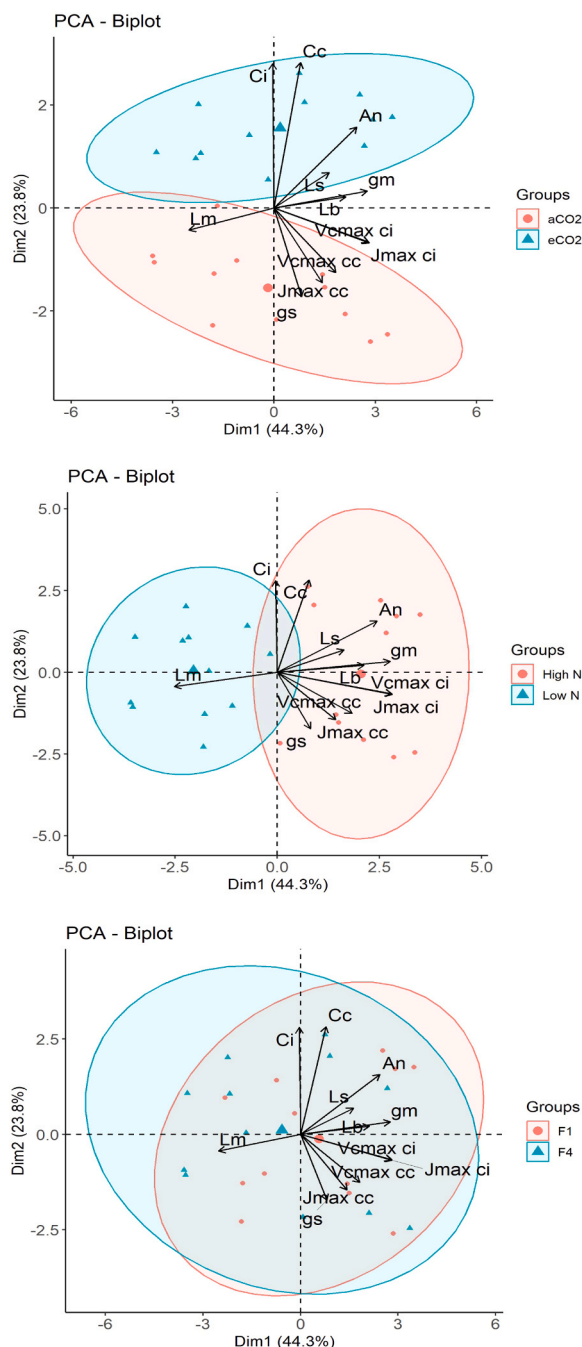


Fig. 6. Plots of principal component analysis (PCA) of plants grown under two atmospheric CO₂ concentrations (400 $\mu\text{mol mol}^{-1}$ and 800 $\mu\text{mol mol}^{-1}$) in the grain filling stage. The contributions of each PCA axis (PC1 and PC2) were indicated on the graph.

varieties with superior NUE and, potentially, greater intrinsic g_m , to fully realize the theoretical benefits of atmospheric CO₂ enrichment for crop productivity.

CRediT authorship contribution statement

Eva Rosenqvist: Methodology. **Xizi Wang:** Formal analysis, Data curation. **Yin Wang:** Methodology. **Weifeng Wang:** Formal analysis,

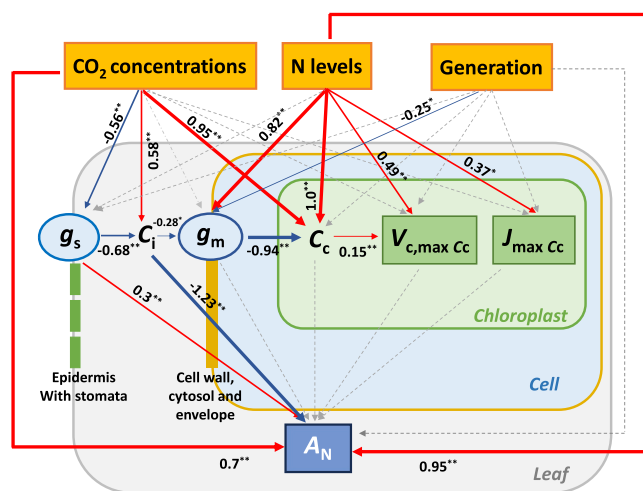


Fig. 7. Maximum likelihood multiple model. Each box in schematics represents observation variables. Path coefficients are reflected by the width of the arrow, with blue solid lines indicating a significantly negative effect, red solid lines indicating a significantly positive effect, and black dotted lines indicating no significant effect. * $P \leq 0.05$, ** $P \leq 0.01$.

Data curation. **Yuzheng Zong:** Writing – original draft, Methodology. **Fulai Liu:** Supervision, Conceptualization. **Jinxiang Hou:** Data curation.

Declaration of Competing Interest

We declare that we have no financial or personal relationships with individuals or organizations that could inappropriately influence our work. There is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in the manuscript entitled.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.envexpbot.2026.106372](https://doi.org/10.1016/j.envexpbot.2026.106372).

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