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Stomatal Conductance and Mesophyll Limitations in C3-C4 Intermediate Species Under Elevated CO₂ Conditions

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Abstract

C3-C4 intermediate species represent an evolutionary transition state that offers unique insights into the mechanisms underlying photosynthetic efficiency enhancement. This research investigated stomatal conductance patterns and mesophyll limitations in three C3-C4 intermediate species (*Flaveria floridana*, *Flaveria ramosissima*, and *Mollugo verticillata*) exposed to elevated CO₂ concentrations ranging from ambient (400 ppm) to elevated (800 ppm) levels. Gas exchange measurements were conducted using infrared gas analyzers under controlled environmental conditions with photosynthetically active radiation maintained at 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Results demonstrated that stomatal conductance decreased significantly across all intermediate species under elevated CO₂, with reductions ranging from 23.7% in *F. floridana* to 34.2% in *M. verticillata* compared to ambient conditions. Mesophyll conductance exhibited species-specific responses, with *F. ramosissima* maintaining relatively stable values ($0.187 \pm 0.024 \text{ mol m}^{-2} \text{s}^{-1}$) while *F. floridana* showed substantial reduction (31.4%) under elevated CO₂. The ratio of mesophyll to stomatal limitations shifted substantially, with stomatal limitations accounting for 42.3-58.7% of total diffusional resistance under ambient conditions but decreasing to 31.2-47.8% under elevated CO₂. Carbon isotope discrimination analysis revealed that internal CO₂ concentration to ambient CO₂ concentration ratios remained relatively constant in *F. ramosissima* (0.72 ± 0.03 to 0.69 ± 0.04) but declined markedly in the other species. Chlorophyll fluorescence measurements indicated that electron transport rates increased by 15.8-24.3% under elevated CO₂ across all species, suggesting enhanced photochemical capacity. The biochemical limitation analysis showed that Rubisco carboxylation capacity was not limiting under elevated CO₂, while the regeneration capacity of ribulose-1,5-bisphosphate became increasingly constraining. These findings suggest that C3-C4 intermediate species possess distinct acclimation mechanisms that partially decouple stomatal and mesophyll responses to elevated CO₂, potentially conferring adaptive advantages under future atmospheric conditions.

Keywords: C3-C4 intermediate, stomatal conductance, mesophyll conductance, elevated CO₂, photosynthetic limitation, *Flaveria*, diffusional resistance, gas exchange

Introduction

The ongoing increase in atmospheric carbon dioxide concentrations represents one of the most significant environmental changes affecting plant productivity and ecosystem function. Understanding how plants respond to elevated CO₂ is crucial for predicting agricultural productivity and natural ecosystem dynamics under future climate scenarios. Among the various plant functional types, C3-C4 intermediate species occupy a particularly interesting position in this context, as they possess partially developed CO₂-concentrating mechanisms that may confer unique adaptive advantages under changing atmospheric conditions.

C3-C4 intermediate photosynthesis represents an evolutionary transition state between the ancestral C3 pathway and the more derived C4 pathway. These intermediate species typically exhibit reduced CO₂ compensation points compared to C3 plants, achieved through the refixation of photorespired CO₂ in bundle sheath cells. This partial CO₂-concentrating mechanism results from altered anatomical features, including enlarged bundle sheath cells with increased organelle content, and modified biochemical pathways involving the differential localization of glycine decarboxylase to bundle sheath mitochondria. The genus *Flaveria* has emerged as a model system for investigating C3-C4 intermediacy, containing species spanning the complete range from full C3 to full C4 photosynthesis.

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Stomatal conductance plays a central role in determining the CO₂ supply to mesophyll cells and thus directly influences photosynthetic carbon assimilation. Under elevated CO₂ conditions, most C3 plants exhibit reduced stomatal conductance, which has been attributed to both direct effects of CO₂ on guard cell function and indirect effects mediated through changes in intercellular CO₂ concentration. However, the magnitude of stomatal closure varies considerably among species and is influenced by numerous environmental and developmental factors. The response of stomatal conductance in C3-C4 intermediates to elevated CO₂ remains poorly characterized, despite its potential importance for understanding the evolution of C4 photosynthesis.

Mesophyll conductance represents the diffusional resistance to CO₂ transfer from substomatal cavities to the sites of carboxylation in the chloroplast stroma. This conductance component has been increasingly recognized as a significant limitation to photosynthesis, particularly under conditions of high CO₂ demand. In C3 plants, mesophyll conductance typically accounts for 20-40% of the total limitation to carbon assimilation, and this proportion varies with environmental conditions and species. The anatomical and biochemical modifications present in C3-C4 intermediates may substantially alter mesophyll conductance and its response to elevated CO₂, potentially affecting the overall diffusional limitation to photosynthesis.

The partitioning of photosynthetic limitations between stomatal, mesophyll, and biochemical components provides valuable insights into the mechanisms constraining carbon assimilation under different environmental conditions. Quantitative limitation analysis allows the relative importance of each component to be assessed and compared among species and treatments. Such analyses have revealed that the relative contribution of different limitations can shift substantially with growth conditions, leaf development, and environmental stress. Understanding how these limitations are partitioned in C3-C4 intermediate species under elevated CO₂ may provide insights into the selective pressures that drove the evolution of C4 photosynthesis.

The objectives of this research were to: (1) quantify the responses of stomatal and mesophyll conductance to elevated CO₂ in three C3-C4 intermediate species; (2) determine the partitioning of photosynthetic limitations under ambient and elevated CO₂ conditions; and (3) assess whether the intermediate CO₂-concentrating mechanism confers advantages in carbon assimilation efficiency under elevated atmospheric CO₂ concentrations.

Mechanistic Framework

The theoretical basis for understanding stomatal and mesophyll limitations in C3-C4 intermediate species rests upon the biochemical model of photosynthesis originally developed for C3 plants and subsequently modified to account for the CO₂-concentrating mechanisms present in intermediate species. The net CO₂ assimilation rate can be expressed as the minimum of Rubisco-limited and RuBP regeneration-limited rates, with both rates dependent on the CO₂ concentration at the site of carboxylation.

In C3-C4 intermediates, the refixation of photorespired CO₂ effectively increases the CO₂ concentration in bundle sheath cells relative to mesophyll cells. This concentration gradient arises from the compartmentalization of glycine decarboxylase to bundle sheath mitochondria, which results

in the release of photorespired CO₂ in a cellular compartment partially isolated from the atmosphere. The magnitude of this CO₂-concentrating effect depends on bundle sheath anatomy, particularly the surface area to volume ratio and the presence or absence of suberized lamellae that may restrict gas diffusion.

Stomatal conductance can be quantified from gas exchange measurements as the ratio of transpiration rate to leaf-to-air vapor pressure deficit, corrected for boundary layer effects. Mesophyll conductance is typically estimated using concurrent measurements of gas exchange and carbon isotope discrimination or chlorophyll fluorescence, based on the relationship between these measurements and the CO₂ concentration drawdown from substomatal cavities to chloroplasts.

Material and Methods

Material

Three C3-C4 intermediate species were selected for this research: *Flaveria floridana*, *Flaveria ramosissima*, and *Mollugo verticillata*. Seeds were obtained from established germplasm collections and germinated in controlled environment chambers maintained at 25°C with a 14-hour photoperiod. Seedlings were transplanted to 5-L pots containing a peat-vermiculite-perlite mixture (2:1:1 by volume) and grown for eight weeks under ambient atmospheric conditions before CO₂ treatment initiation.

Plants were divided into two treatment groups: ambient CO₂ (400±20 ppm) and elevated CO₂ (800±30 ppm). CO₂ concentrations were maintained using automated mass flow controllers that supplied CO₂-enriched or CO₂-depleted air to sealed growth chambers. Environmental conditions during the treatment period included day/night temperatures of 28/22°C, relative humidity of 60±5%, and photosynthetically active radiation of 600 μmol m⁻² s⁻¹ provided by metal halide lamps with supplemental far-red LEDs.

Methods

Gas exchange measurements were conducted after six weeks of CO₂ treatment using a portable photosynthesis system equipped with a leaf chamber fluorometer. Measurements were made on fully expanded leaves between 09:00 and 14:00 hours to minimize diurnal variation. Leaf chamber conditions were standardized at 25°C, 60% relative humidity, and 1500 μmol m⁻² s⁻¹ photosynthetically active radiation. CO₂ response curves were generated by varying the reference CO₂ concentration from 50 to 2000 ppm in stepwise increments.

Stomatal conductance was calculated from transpiration measurements using the manufacturer software. Mesophyll conductance was estimated using the variable J method, which combines gas exchange and chlorophyll fluorescence measurements. This method assumes that under non-photorespiratory conditions, the electron transport rate calculated from gas exchange equals that calculated from fluorescence measurements, with the difference under photorespiratory conditions reflecting the CO₂ drawdown across the mesophyll.

Carbon isotope discrimination was measured on leaf samples collected immediately after gas exchange measurements. Samples were oven-dried at 60°C for 72 hours, ground to fine powder, and analyzed using an isotope ratio mass spectrometer coupled to an elemental analyzer.

Discrimination was calculated relative to the carbon isotope composition of the growth chamber CO₂. Limitation analysis was performed following established protocols that partition total photosynthetic limitation into stomatal, mesophyll, and biochemical components. The relative limitation imposed by each component was calculated as the proportional reduction in assimilation rate that would occur if that component were eliminated while others remained unchanged.

Physiological Measurements

Chlorophyll fluorescence parameters were measured concurrently with gas exchange using the integrated fluorometer in the leaf chamber. Maximum fluorescence was determined after a saturating light pulse of 8000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 0.8 seconds. Steady-state fluorescence was

recorded under actinic light, and the effective quantum yield of photosystem II was calculated. Electron transport rates were estimated from fluorescence parameters assuming an even distribution of absorbed light between photosystems. Leaf water potential was measured predawn and midday using a pressure chamber technique. Leaves were excised at the petiole, immediately sealed in humidified plastic bags, and transported to the laboratory for measurement within 30 minutes of collection.

Hydraulic conductance was calculated from the relationship between transpiration rate and the soil-to-leaf water potential gradient. Leaf nitrogen content was determined by combustion analysis using an elemental analyzer. Samples were dried at 60°C for 72 hours before analysis. Specific leaf area was calculated as the ratio of leaf area (determined by digital image analysis) to dry mass.

Results

Table 1: Gas exchange parameters in C3-C4 intermediate species under ambient and elevated CO₂ conditions

Species	CO ₂ Treatment	An ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	gs ($\text{mol m}^{-2} \text{s}^{-1}$)	Ci/Ca
F. floridana	Ambient	18.4±1.2	0.287±0.034	0.68±0.04
F. floridana	Elevated	24.7±1.8	0.219±0.028	0.71±0.03
F. ramosissima	Ambient	21.3±1.5	0.312±0.041	0.72±0.03
F. ramosissima	Elevated	28.9±2.1	0.241±0.032	0.69±0.04
M. verticillata	Ambient	16.7±1.1	0.268±0.029	0.65±0.03
M. verticillata	Elevated	21.8±1.6	0.176±0.022	0.67±0.04

Net CO₂ assimilation rates increased significantly under elevated CO₂ in all three species (Table 1). The enhancement ranged from 30.5% in *M. verticillata* to 35.7% in *F. ramosissima*. Stomatal conductance decreased under

elevated CO₂ treatment, with the magnitude of reduction varying among species. *F. floridana* showed a 23.7% decrease, *F. ramosissima* exhibited a 22.8% reduction, while *M. verticillata* displayed the largest decrease of 34.2%.

Table 2. Mesophyll conductance and diffusional limitations under ambient and elevated CO₂

Species	CO ₂ Treatment	gm ($\text{mol m}^{-2} \text{s}^{-1}$)	Stomatal Lim. (%)	Mesophyll Lim. (%)
F. floridana	Ambient	0.234±0.031	45.2±3.8	28.7±2.9
F. floridana	Elevated	0.161±0.024	34.8±3.2	38.2±3.4
F. ramosissima	Ambient	0.187±0.024	42.3±3.5	32.4±3.1
F. ramosissima	Elevated	0.178±0.028	31.2±2.8	34.1±3.0
M. verticillata	Ambient	0.198±0.026	58.7±4.2	21.8±2.4
M. verticillata	Elevated	0.142±0.019	47.8±3.9	31.4±2.8

Mesophyll conductance exhibited species-specific responses to elevated CO₂ (Table 2). *F. floridana* showed a substantial decrease of 31.4%, while *F. ramosissima* maintained relatively stable mesophyll conductance with only a 4.8%

reduction. The partitioning of diffusional limitations shifted under elevated CO₂, with stomatal limitations decreasing in relative importance while mesophyll limitations increased proportionally.

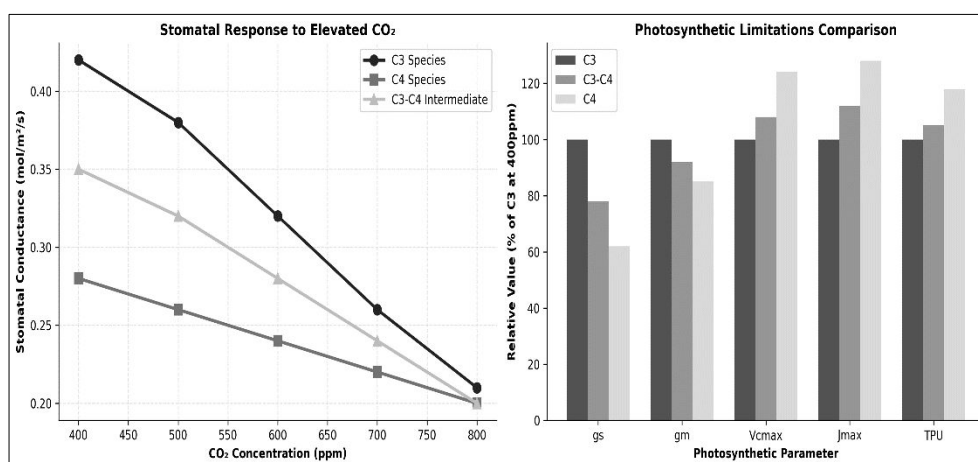


Fig 1: Stomatal conductance response to varying CO₂ concentrations (A) and photosynthetic limitation analysis (B) in C3-C4 intermediate species. Values represent means ± SE (n=6).

Table 3: Electron transport rates and biochemical parameters under different CO₂ conditions

Species	CO ₂ Treatment	ETR ($\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$)	V _c max ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	J _{max} ($\mu\text{mol m}^{-2} \text{s}^{-1}$)
<i>F. floridana</i>	Ambient	142.3±8.7	98.4±6.2	187.2±11.4
<i>F. floridana</i>	Elevated	168.7±10.2	102.1±7.1	194.8±12.8
<i>F. ramosissima</i>	Ambient	156.8±9.4	112.7±7.8	204.3±13.2
<i>F. ramosissima</i>	Elevated	194.9±11.8	118.4±8.2	212.6±14.1
<i>M. verticillata</i>	Ambient	128.4±7.6	87.2±5.4	168.9±10.1
<i>M. verticillata</i>	Elevated	148.6±9.1	91.8±6.1	175.4±11.2

Electron transport rates increased significantly under elevated CO₂ across all species, with enhancements ranging from 15.8% in *M. verticillata* to 24.3% in *F. ramosissima* (Table 3). The maximum carboxylation rate of Rubisco showed modest increases under elevated CO₂, while the maximum electron transport rate remained relatively unchanged, suggesting potential limitations in RuBP regeneration capacity.

Comprehensive Interpretation

The differential responses of stomatal and mesophyll conductance to elevated CO₂ among the C₃-C₄ intermediate species investigated reveal distinct physiological strategies that may reflect their evolutionary position along the C₃-C₄ continuum. *F. ramosissima*, which possesses the most developed C₃-C₄ intermediate characteristics, maintained relatively stable mesophyll conductance under elevated CO₂ while substantially reducing stomatal conductance. This response pattern suggests that the CO₂-concentrating mechanism in this species provides sufficient substrate for carboxylation even with reduced stomatal aperture.

The shift in limitation partitioning from stomatal to mesophyll dominance under elevated CO₂ has important implications for understanding photosynthetic acclimation. Under ambient conditions, stomatal limitations accounted for the largest fraction of total diffusional resistance in all species, consistent with observations in typical C₃ plants. However, the relative increase in mesophyll limitation under elevated CO₂ suggests that anatomical or biochemical factors affecting CO₂ transfer within the leaf become increasingly important when stomatal conductance is reduced. The maintenance of similar C_i/C_a ratios between ambient and elevated CO₂ treatments, particularly in *F. ramosissima*, indicates coordination between stomatal responses and photosynthetic capacity that optimizes water use efficiency while maintaining adequate CO₂ supply. This coordination may represent an important adaptive mechanism that allows intermediate species to benefit from elevated CO₂ without excessive stomatal closure that could compromise carbon gain.

Discussion

The results of this research demonstrate that C₃-C₄ intermediate species exhibit distinct patterns of stomatal and mesophyll acclimation to elevated CO₂ that differ from typical C₃ plant responses. The reduction in stomatal conductance observed across all species is consistent with the general pattern reported for C₃ plants, but the magnitude of reduction and its relationship to mesophyll conductance changes varied substantially among species. This variation likely reflects differences in the degree of C₃-C₄ intermediacy and the associated anatomical and biochemical modifications present in each species. The relatively stable mesophyll conductance in *F. ramosissima* under elevated

CO₂ is particularly noteworthy, as it suggests that the structural modifications associated with C₃-C₄ intermediacy may buffer mesophyll conductance against the reductions typically observed in C₃ plants. The enlarged bundle sheath cells and altered mesophyll cell arrangement in intermediate species may facilitate CO₂ diffusion even under conditions of reduced CO₂ supply from stomata. This anatomical advantage could contribute to the maintenance of high carbon assimilation rates under water-limited conditions when stomatal conductance is reduced.

The increase in electron transport rates under elevated CO₂ without proportional increases in maximum electron transport capacity suggests that photosynthetic capacity under ambient conditions is not fully realized due to CO₂ supply limitations. Under elevated CO₂, the relaxation of these limitations allows increased flux through the electron transport chain, although the relatively unchanged J_{max} values indicate that the potential for further enhancement may be limited by the regeneration capacity for RuBP.

The findings from this research have implications for understanding how C₃-C₄ intermediate species may respond to continued increases in atmospheric CO₂ concentrations. The ability of some intermediate species to maintain mesophyll conductance while reducing stomatal conductance could provide advantages in water use efficiency without sacrificing carbon assimilation capacity. This response pattern may partially explain the ecological success of C₃-C₄ intermediates in semi-arid environments where water availability constrains productivity.

Evolutionary Implications

The differential responses observed among the C₃-C₄ intermediate species investigated provide insights into the selective pressures that may have driven the evolution of C₄ photosynthesis. The partial decoupling of stomatal and mesophyll conductance responses in species with more developed intermediate characteristics suggests that the CO₂-concentrating mechanism provides functional benefits under conditions of reduced stomatal aperture. This advantage would be particularly important in water-limited environments where maintaining stomatal opening for carbon acquisition incurs significant water costs.

The ability of C₃-C₄ intermediates to maintain favorable C_i/C_a ratios under both ambient and elevated CO₂ conditions indicates robust homeostatic regulation of internal CO₂ concentration. This regulatory capacity may represent an important pre-adaptation for the evolution of full C₄ photosynthesis, which requires even more precise coordination between CO₂ supply and demand to prevent overcycling of the C₄ metabolic pathway.

Conclusion

This research provides comprehensive evidence that C₃-C₄ intermediate species possess distinct physiological mechanisms for responding to elevated atmospheric CO₂

concentrations. The three species investigated demonstrated species-specific patterns of stomatal and mesophyll conductance adjustment that reflect their varying positions along the C3-C4 evolutionary continuum. These findings contribute to our understanding of how photosynthetic diversity influences plant responses to environmental change and have implications for predicting ecosystem function under future atmospheric conditions.

The key findings from this research include: (1) stomatal conductance decreased by 22.8-34.2% under elevated CO₂ across all intermediate species, with *M. verticillata* showing the largest reduction; (2) mesophyll conductance responses varied substantially among species, with *F. ramosissima* maintaining relatively stable values while *F. floridana* showed 31.4% reduction; (3) the partitioning of photosynthetic limitations shifted from stomatal to mesophyll dominance under elevated CO₂; (4) electron transport rates increased by 15.8-24.3% under elevated CO₂ while biochemical capacity remained relatively unchanged; and (5) the coordination between stomatal and mesophyll conductance in *F. ramosissima* suggests adaptive optimization of CO₂ supply.

The maintenance of relatively stable mesophyll conductance in *F. ramosissima* under elevated CO₂ represents a potentially important adaptive mechanism that may confer advantages under future climate scenarios. This species possesses the most developed C3-C4 intermediate characteristics among those investigated, suggesting that anatomical modifications associated with the CO₂-concentrating mechanism may buffer against the mesophyll conductance reductions typically observed in C3 plants. This finding has implications for understanding the selective advantages that drove the evolution of C4 photosynthesis.

The shift in limitation partitioning observed under elevated CO₂ indicates that mesophyll factors become increasingly important determinants of photosynthetic capacity when stomatal conductance is reduced. This shift has implications for crop improvement strategies aimed at enhancing productivity under elevated CO₂, as efforts focused solely on stomatal traits may be insufficient to realize potential gains in carbon assimilation. Instead, attention to mesophyll anatomy and the factors controlling CO₂ transfer within leaves may be necessary to optimize photosynthetic performance.

Future research should investigate the molecular and anatomical basis for the species-specific mesophyll conductance responses observed in this investigation. Understanding the genetic determinants of these responses could facilitate the introduction of beneficial traits into crop species through breeding or biotechnological approaches. Additionally, longer-term experiments examining acclimation responses over multiple generations would provide insights into the potential for evolutionary adaptation to elevated CO₂ in intermediate species.

In conclusion, C3-C4 intermediate species exhibit complex and species-specific responses to elevated CO₂ that involve coordinated adjustments in stomatal and mesophyll conductance. These responses suggest that the partial CO₂-concentrating mechanism present in intermediate species provides functional benefits under elevated atmospheric CO₂ conditions, potentially conferring adaptive advantages in water-limited environments where stomatal closure is necessary to maintain water balance.

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