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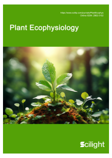

Review

C_2 Photosynthesis across Scales

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Abstract: Stomata patterning, leaf structure, gas exchange, and ultimately species biogeography are inherently connected and have been observed to differ in plants by photosynthetic type. Patterns distinguishing C_3 and C_4 plants across scales are elucidated well in the literature. However, how trait shifts occur along the C_3 to C_4 evolutionary continuum are less clear. This review focuses on the role of C_2 species as C_3-C_4 evolutionary intermediates that help to transition important traits from typical C_3 to C_4 phenotypes across scales. We show that the C_2 phenotype is remarkably diverse and not always intermediate between C_3 and C_4 phenotypes. We highlight that studies of C_2 species in the literature commonly lump C_3-C_4 evolutionary intermediates, stable state C_2 species, and C_3 × C_4 hybrid species together and we argue that these different C_2 types should be distinguished, when possible, and researched independently to help explain the broad diversity of C_2 phenotypes and their global biogeography.

Keywords: biogeography; C_2 photosynthesis; C_3-C_4 intermediate; C_4 photosynthesis evolution; carbon concentrating mechanism; gas exchange; leaf anatomy; stomata

1. The Rise of C_2 Biochemistry

In plants using C_3 photosynthesis, the most common and ancestral type of photosynthesis, the Calvin-Benson-Bassham (CBB) cycle occurs within a single cell type- usually the leaf mesophyll- when RuBP (ribulose-1,5-bisphosphate) binds CO_2 to Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) (Figure 1A). Plants that use only C_3 photosynthesis tend to excel under temperate environments, but suffer under hot, dry, and saline conditions in which stomatal pores on their leaf surface close to minimise water loss, which simultaneously decreases CO_2 influx and eventually CO_2 concentrations within the internal leaf air space (Cowan, 1982). The resulting decrease in the CO_2:O_2 ratio within the leaf promotes the oxygenation of Rubisco which evolves 2-phosphoglycolate, a toxic product that inhibits enzymes central to carbon metabolism (Leegood, 2007). The photorespiratory pathway effectively recycles and detoxifies this 2-phosphoglycolate, but at an energetic and metabolic cost, releasing most of this previously fixed CO_2 into the atmosphere in the process to ultimately decrease net CO_2 assimilation and, therefore, photosynthetic efficiency (Leegood et al., 1995; Tolbert 1980).

Plant lineages have repeatedly and convergently evolved carbon concentrating mechanisms (CCMs) to minimise the costs of photorespiration. For example, in C_4 photosynthesis, a CCM largely avoids photorespiration by initially assimilating CO_2 via PEPC (phosphoenolpyruvate carboxylase)–an enzyme that does not bind to oxygen–rather than via Rubisco. PEPC fixes CO_2 in the mesophyll then shuttles it into the bundle sheath compartment, where CO_2 concentrates and is assimilated via Rubisco into the CBB cycle in a high CO_2:O_2 environment. Thus, through a combination of biochemical and anatomical modifications that facilitate a two-cell CCM, C_4 photosynthesis effectively minimizes photorespiration to boost photosynthetic efficiency under warm, dry, and saline environments that promote high rates of photorespiration (Dai, Ku, & Edwards, 1993). The C_4 trait is highly convergent, with versions of the C_4 CCM having evolved independently in over 70 plant lineages (RF Sage, Christin, & Edwards, 2011).

The evolutionary transitions between the ancestral C_3 and complex C_4 states occurred via a series of discrete intermediate stages (RF Sage, Khoshravesh, & TL Sage, 2014; Leung et al., 2024). Many of these C_3-C_4 evolutionary intermediates use a rare CCM called C_2 photosynthesis, which has also been called the glycine shuttle or photorespiratory CO_2 pump (hereafter referred to as C_2 photosynthesis). The C_2 CCM is currently known to be used by only about 70 species from at least 22 independent plant lineages across 13 families (Figure 2, Table 1), including some in the economically important crop families Amaranthaceae, Asteraceae, Brassicaceae, and Poaceae.

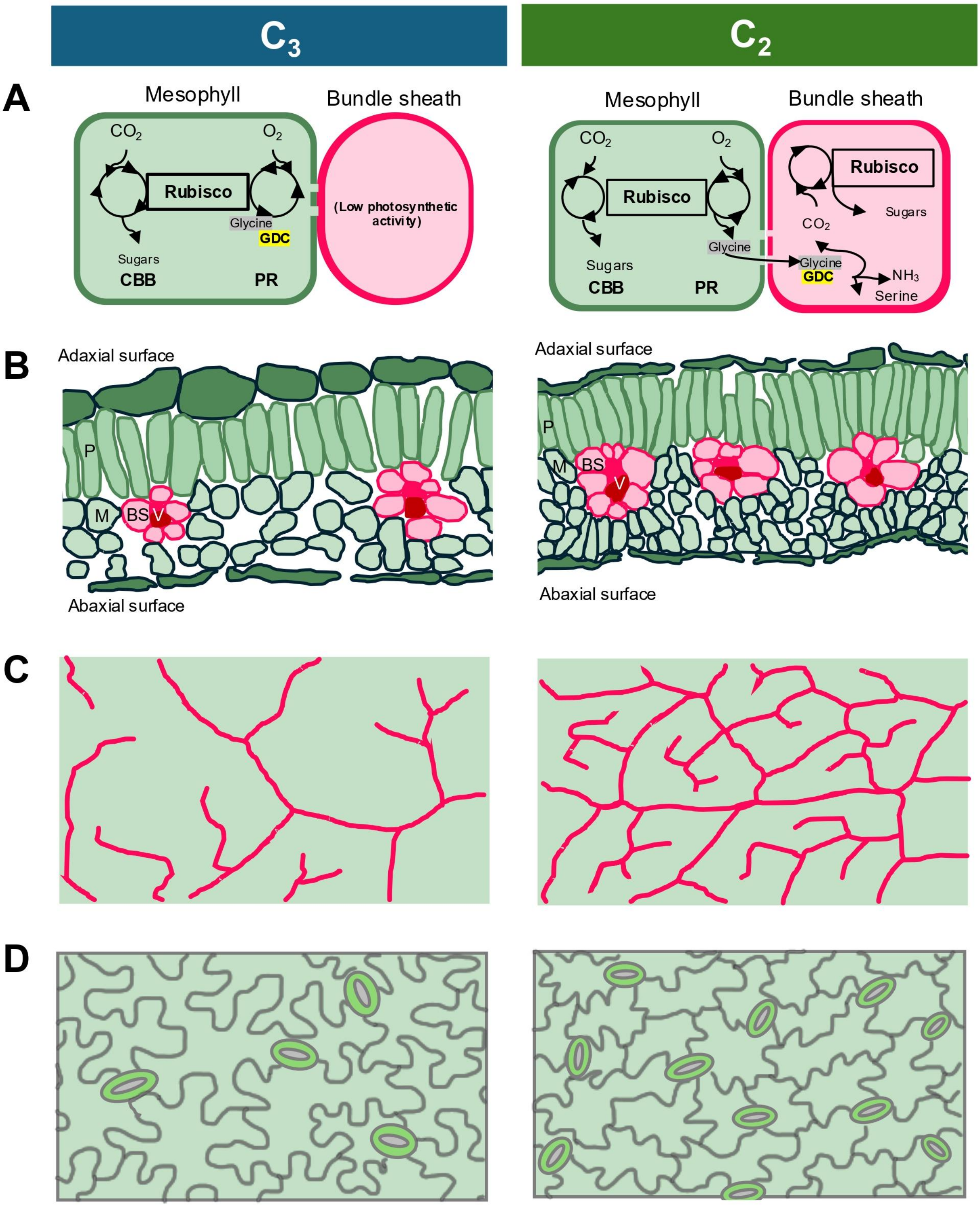

Figure 1. Comparison of C_3 and C_2 photosynthesis across scales. (**A**) C_3 photosynthesis takes place within a single cell type, primarily within mesophyll cells. In contrast, photorespiratory glycine diffuses via a concentration gradient from mesophyll (M) to bundle sheath (BS) cells in C_2 plants, which have enhanced M:BS surface contact to increase diffusion of photosynthate and glycine between the two cell types. GDC, glycine decarboxylase complex. (**B**) Structurally, interveinal tends to be smaller in C_2 leaves, with fewer mesophyll cells between veins, compared to C_3 leaves; V, vein, P, palisade mesophyll, M, spongy mesophyll, BS, bundle sheath. (**C**) Vein density and (**D**) stomatal density have been observed to be higher in C_2 compared to C_3 leaves. Cartoons in panels B, C, and D have been traced from actual *Parthenium incanum* (C_3) and *Parthenium hysterophorus* (C_2) leaves in (**A**) cross sections, (**B**) cleared paradermal sections, and (**C**) epidermal imprints.

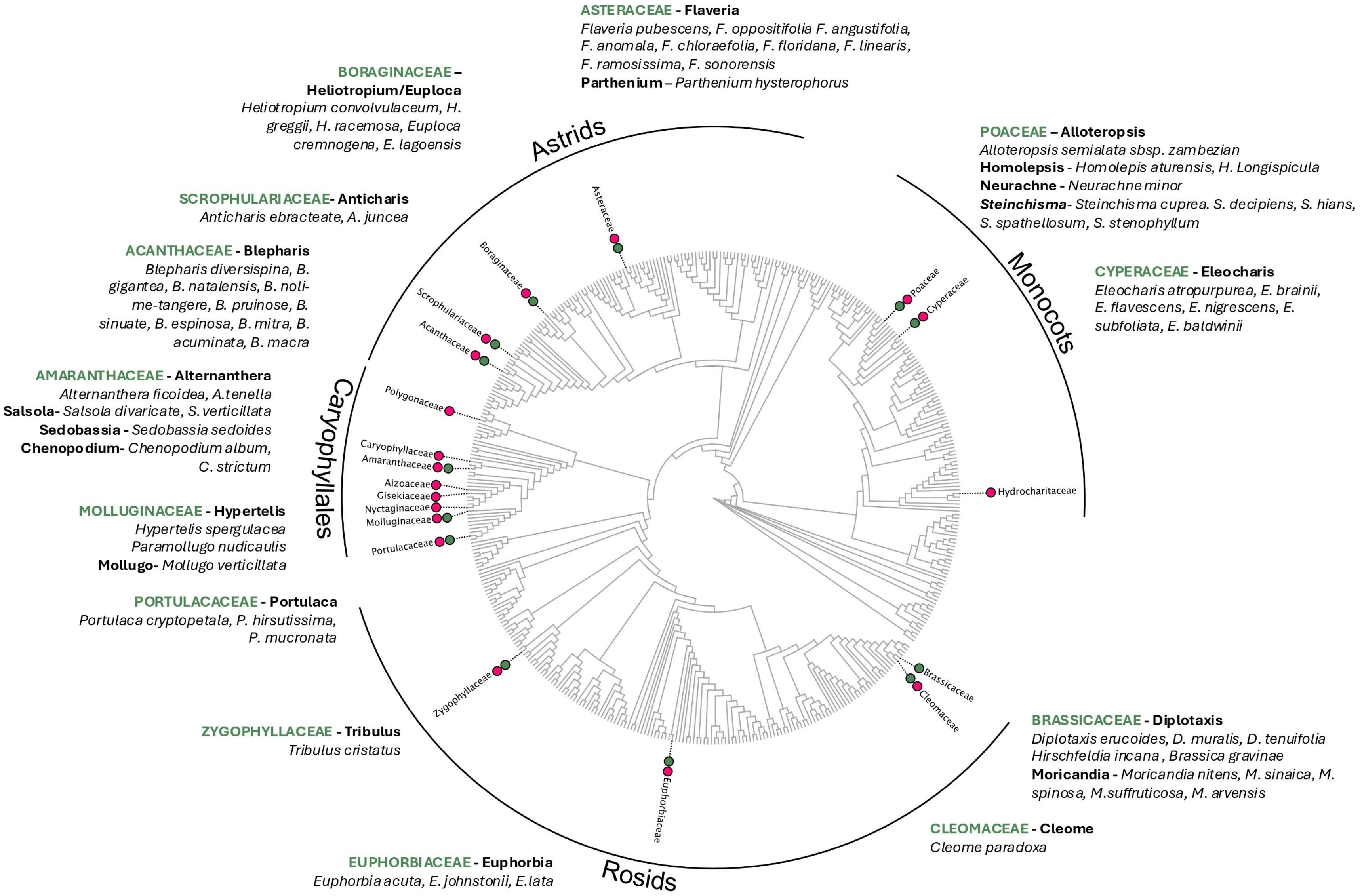

Figure 2. Distribution of C_2 and C_4 plant families across the angiosperms. Plant families that have evolved C_2 (green) or C_4 (red) carbon concentrating mechanisms are plotted on the Plant and Fungal Trees of Life Project (PAFTOL) angiosperm phylogeny from Zuntini et al. (2024). The phylogeny was constructed using the *ggtree* and *ape* R packages (Yu et al., 2017; Paradis & Schliep, 2019). All C_2 species are listed within their family.

Table 1. Defining three distinct types of C_2 plants.

C_2 Type	Definition	References
C_3-C_4 evolutionary intermediate species	All plants using a C_2 photosynthesis glycine shuttle were originally believed to be C_3-C_4 evolutionary intermediates, that is, a transitional stage during the evolution from the ancestral C_3 to the derived C_4 photosynthetic phenotype. Their role as an evolutionary intermediate stepping stone to C_4 makes sense, as C_2 species often present with anatomical and physiological phenotypes that fall intermediate between C_3 and C_4 close relatives and modelling explains that the C_2 photosynthesis glycine shuttle would almost inevitably encourage C_4 emergence. C_3-C_4 intermediate C_2 species may engage weak C_4 cycle activity. The majority of currently characterised C_2 species are C_3-C_4 intermediates.	Holaday & Chollet 1984; Monson, Edwards, & Ku, 1984; Heckmann et al., 2013; RF Sage, Khoshravesh, & TL Sage, 2014
Stable-state C_2 species	Recently termed 'super C_2', stable-state C_2 plants appear to have a particularly efficient CCM system that presents with very low CO_2 compensation points (e.g., aligning with values typically measured in C_4 plants) and very negative $\delta^{13}C$ signatures that completely rule out C_4 activity. These species tend to have broad ecological and geographical ranges. Such lineages remain in the C_2 stage, rather than evolving toward a C_4 phenotype. Stable-state C_2 species usually lack close C_4 relatives. Only a handful of stable-state C_2 species have been characterised to date.	Lundgren 2020; Christin et al., 2011; Lundgren & Christin 2017; Blätke & Bräutigam, 2019; Siadjeu, Lauterbach, & Kadereit, 2021; Walsh et al., 2023
$C_3 \times C_4$ hybrid species	$C_3 \times C_4$ hybrid species or individual plants arose via hybridisation of C_3 and C_4 parent lineages at some point in the past. These C_2 species can present with some degree of C_4 traits, as inherited. $C_3 \times C_4$ hybrid individuals can be inter-specific (e.g., *Salsola*) or intra-specific (e.g., *Alloteropsis*). $C_3 \times C_4$ hybrid C_2 species are currently believed to be rare, but more research is needed.	Monson, Edwards, & Ku, 1984; Kadereit et al., 2017; Alvarenga et al., 2025; Tefarikis et al., 2022; Dunning et al., 2017

The C_2 CCM boosts photosynthetic efficiency by capturing and reassimilating CO_2 released by photorespiration. To do this, C_2 plants spread the photorespiratory cycle across mesophyll and bundle sheath cells by restricting glycine decarboxylase complex (GDC) activity to the bundle sheath, such that glycine produced by photorespiration accumulates in the mesophyll until the concentration gradient causes glycine to diffuse into the bundle sheath where, upon encountering GDC, it is broken down into CO_2, NH_3, and serine (Figure 1A). The released CO_2 concentrates and is reassimilated via the CBB cycle within bundle sheath cells. The C_2 photosynthetic system, therefore, reassimilates CO_2 released from mesophyll photorespiration while simultaneously concentrating CO_2 in the bundle sheath to decrease photorespiration in that cell type, which together effectively increase net carbon assimilation. The C_2 CCM effectively maintains the important functions that photorespiration provides, including links with respiration, the TCA cycle (tricarboxylic acid cycle), and carbon, nitrogen, sulphur, and C1 metabolisms (e.g., Timm and Bauwe, 2013; Busch, RF Sage, & Farquhar, 2018; Eisenhut, Roell, & Weber, 2019). Indeed, photorespiratory nitrogen release is believed to be central to the emergence of both C_2 and C_4 photosynthetic innovations (Mallmann et al., 2014; Bräutigam & Gowik, 2016; Adachi et al., 2023). Thus, C_2 species can have varying degrees of weak C_4 activity, depending upon where they fall along the C_3 to C_4 evolutionary continuum. These C_2 species that engage weak C_4 enzyme activity were previously referred to as "C_2 Type II" species but are now generally termed C_2+ (Leung et al., 2024; Alvarenga et al., 2025). Indeed, C_2+ species actively express C_4 cycle enzymes (Dunning et al., 2019; Adachi et al., 2023; Lyu et al., 2023; Leung et al., 2024; Stata et al., 2025).

Because C_2 plants can engage varying degrees of C_3, C_2, and sometimes C_4 activity within the same leaf, they cannot uniformly be identified using stable $\delta^{13}C$ isotopes. Thus, while $\delta^{13}C$ signatures are an excellent tool to distinguish C_3 (e.g., $< \sim-25‰$) from C_4 (e.g., $> \sim-15‰$) plants (O'Leary 1981; Ubierna, Holloway-Phillips, & Farquhar, 2018a, 2018b), they can only be used to partially explain the underlying physiology of C_2 plants (Caemmerer 1992). For example, C_2 plants that lack C_4 cycle activity will have very negative $\delta^{13}C$ signatures (e.g., $< -35‰$) due to their double-discrimination of the ^{13}C isotope. In contrast, C_2 plants that engage large amount of C_4 cycle activity will have $\delta^{13}C$ signatures that fall intermediate between that typical of C_3 and C_4 plants (e.g., between −15‰ and −25‰), which reflects the amount of carbon that is initially fixed via the PEPC, rather than Rubisco (Monson et al., 1988; Lundgren et al., 2016). However, C_2 leaf tissue that did not strongly engage the glycine shuttle (e.g., because the growth environment did not induce high rates of photorespiration) or strongly engage C_4 cycle activity (e.g., because the C_4 CCM is too weak) will present with $\delta^{13}C$ signatures that completely overlap with C_3 plants (i.e., between −35 and −25‰) (Edwards & Ku, 1987).

2. Distinguishing Three Types of C_2 Plants

C_2 photosynthesis has been largely studied in the context of C_4 evolution, however, not all C_2 species fall into these described patterns of C_3-C_4 intermediacy (Tables 1 and 2; Holaday & Chollet 1984; Monson, Edwards, & Ku, 1984). First, although it has been hypothesized that most C_2 species arose primarily via natural selection for carbon and/or nitrogen assimilation benefits, as described above, the C_2 phenotypes can also arise via hybridisation between C_3 and C_4 parents (Monson, Edwards, & Ku, 1984; Mallman et al., 2014; Bräutigam & Gowik, 2016; Kadereit et al., 2017; Adachi et al., 2023). This hybridization process can accelerate the evolution of C_4 metabolism through the introduction of C_4 genes into non-C_4 plants, while also generating intermediate phenotypes that do not reflect gradual evolutionary stages (Kadereit et al., 2017; Alvarenga et al., 2025). For example, Tefarikis et al. (2022) examined carbon isotope discrimination, CO_2 compensation point, PEPC activity, and phylogenetic signals from gene trees and networks to determine that the C_2 species *Salsola divaricata* agg. (Amaranthaceae) arose via an ancient $C_3 \times C_4$ hybridisation event. Similarly, the grass *Alloteropsis semialata* creates $C_3 \times C_4$ interspecific hybrids in nature (Dunning et al., 2017) and in the glasshouse (Bianconi et al., 2022). The grass genus *Homolepis* provides yet another interesting case of photosynthetic diversity emergence whereby the sub-C_2 phenotype in *Homolepis isocalycia* arises from hybridization between non-C_4 species, involving allopolyploidy and gene flow, but not $C_3 \times C_4$ crosses, which indicates that the C_2 state can emerge de novo, without C_4 introgression (Alvarenga et al., 2025).

Some C_2 lineages are not associated with C_4 photosynthesis altogether (Figure 2). Here, C_2 phenotypes emerged from ancestral C_3 ancestors and remained in a C_2 state for long periods of time, without further progression toward C_4 (Table 1). These C_2 lineages include *Parthenium* (Asteraceae), *Mollugo* (Molluginaceae), *Steinchisma* (Poaceae), *Diplotaxis* (Brassicaceae), and *Moricandia* (Brassicaceae) (Table 2; Christin et al., 2011, Lundgren & Christin 2017). The existence of these independent C_2 lineages suggests that some version of the C_2 phenotype is a stable evolutionary state (Lundgren, 2020; Blätke & Bräutigam, 2019; Siadjeu, Lauterbach, & Kadereit, 2021). Indeed, these stable state C_2 species appear to be particularly robust, with broader ecological and geographical ranges than C_3-C_4 intermediate C_2 species (Lundgren & Christin 2017), leading researchers to distinguish them as 'super C_2' species (Table 1; Walsh et al., 2024).

Discussions of C_2 species in the literature currently lump these three distinct types of C_2 phenotypes together. We propose that C_3-C_4 evolutionary intermediate, stable state, and $C_3 \times C_4$ hybrids (which can also present as a stable evolutionary state) should be distinguished, when possible, and researched independently (summarized in Table 1; Figure 2). These different origins of C_2 species likely explain the broad diversity in C_2 phenotypes and biogeography.

3. Stomatal Traits in C_2 Plants

Stomata open and close in response to environmental stimuli, such as water and CO_2 concentration gradients, making stomata the gatekeepers of gas exchange in plants that strongly influence photosynthetic efficiency (Lawson & Blatt 2014; Wang et al., 2022). A combination of traits, including stomatal density, size, index (i.e., the ratio of stomata to the total number of epidermal cells and stomata on a leaf surface), ratio (i.e., the ratio of stomata frequency between the abaxial and adaxial surfaces of the leaf), and speed of reaction interact to regulate the efficacy of gas exchange between the atmosphere and internal leaf environment. Shifts in these individual stomatal traits accompanied the evolutionary transitions between C_3 and C_4 phenotypes (Way, 2012).

C_4 plants function efficiently at low internal CO_2 concentrations, as such, can maintain higher rates of CO_2 assimilations at lower stomatal conductance than C_3 plants, yielding them higher water use efficiency (Raven & Ramsden 1988). Thus, stomatal traits have shifted in plant lineages that evolved C_4 photosynthesis to accommodate the distinct patterns of gas exchange used by C_4 plants. For example, lower rates of stomatal conductance measured in C_4 compared to closely related C_3 grasses (Taylor et al., 2010; 2011) is facilitated by the 40 per cent lower stomatal density and 29 per cent lower maximum stomatal conductance to water vapour (g_{max}) measured in phylogenetically paired C_3 *versus* C_4 relatives across seven grass lineages originating from diverse habitats (Taylor et al., 2012) and the faster stomatal response speeds measured in C_4 compared C_3 monocot and eudicot species (McAusland et al., 2016; Israel et al., 2022; Tanigawa et al., 2024). Despite these clear patterns, evolutionary history plays an important role in stomatal patterning (Edwards, Still, & Donoghue, 2007; Edwards & Still, 2008; Taylor et al., 2010, 2012) such that the evolutionary path to achieve C_4 stomatal patterning can be achieved differently with each independent emergence of C_4 photosynthesis.

The remarkable intraspecific photosynthetic diversity present in the grass *Alloteropsis semialata* offers a unique opportunity to look at the evolutionary transitions along the C_3 to C_4 continuum in detail within a non-domesticated, monocot lineage (Lundgren et al., 2016; Pereira et al., 2023). For example, Bianconi et al., (2022) found that stomatal density was lower in both C_2 and C_4 compared to C_3 accessions of *A. semialata*. More recently, Zhou and Osborne (2024) showed that stomatal response speeds were slower in C_2 and C_4 *A. semialata* accessions compared to their C_3 conspecifics, as a result of changes to guard and subsidiary cell size and higher guard cell volumes in those accessions using CCMs. Specifically, they show that C_2 emergence was associated with stomatal opening speeds, while C_4 emergence was accompanied by the shrinking of both guard and subsidiary cells. Together, these studies suggest that the C_2 phenotype functioned to decrease stomatal density and speed along the evolutionary trajectory toward the C_4 phenotype in this monocot lineage.

Table 2. C_2 lineage growth and habitat preferences. C_2 lineages are presented by family and include the number of C_2 species within each lineage and their proposed type (i.e., C_3-C_4 intermediate, $C_3 \times C_4$ hybrid, and stable state C_2), as updated from Lundgren and Christin 2017. Climatic zone, altitude, optimal growth temperature, moisture preference, and habitat descriptions are provided for each C_2 lineage. Life history is described as annual, subshrub, or perennial. Climatic zone and altitudinal ranges were taken from occurrence data from the Global Biodiversity Information Facility (GBIF, accessed on 21 August 2024), and extracted using the *rgbif* package (Chamberlain et al., 2023). Dominant latitudinal ranges are described as tropical (0–23.5°), subtropical (23.5–40°), or temperate (40–60°). Altitudinal ranges are described as low (0–2000 m above sea level), medium (2000–3500 m), or high (>3500 m). Optical growth temperatures, based on the range of the Bioclim 8 variable ‘mean temperature of wettest quarter’, are described as psychrophiles (growing best in low temperatures between 0 and 10 °C), mesophyles (preferring moderate temperatures and thriving between 10 and 30 °C), or thermophyles (thriving at high temperatures between 30 and 65 °C). Wetness preferences are described as arid (< 200 mm annual precipitation, Bioclim 12), semi-arid (200–400 mm), semi-humid (400–800 mm), and humid (>800 mm). Optimal growth temperature and wetness preference descriptions are based on WorldClim version 2.1 climate data for 1970–2000 Bioclimatic variables (Fick & Hijmans, 2017) extracted and ranges summarised for C_2 species within each lineage using the *terra* and *geodata* R packages (Hijmans, 2023; Hijmans et al., 2024). Habitat descriptions are taken from the literature, as cited.

	Lineage	No. C_2 Species	Hypothesized C_2 Type	Life History	Climatic Zone	Alt	Optimal Growth Temperature	Moisture Preferences	Habitat Descriptions
EUDICOTS									
Acanthaceae	*Blepharis*	10	C_3-C_4 Intermediate	subshrub	Tropical, subtropical	Low	Mesophyles	Arid to humid	Various habitats; predominantly sandy to stony soils, deciduous woodlands, and scrubland (Lundgren & Christin, 2017).
Amaranthaceae	*Alternanthera*	2	C_3-C_4 Intermediate	Annual, clonal	Tropical, subtropical	Low	Mesophyles & Thermophyles	Semi-humid to humid	Various habitats; predominantly disturbed soils and wetlands (Sanchez-Del Pino, Motley, & Borsch, 2012).
	Chenopodium	2	C_3-C_4 Intermediate	Annual	Tropical, subtropical, temperate	Low	Psychrophiles & Mesophyles & Thermophyles	Arid to humid	Various habitats; predominantly disturbed and open, sometimes saline soils (Yorimitsu et al., 2019).
	Salsola	2	$C_3 \times C_4$ hybrid	Annual	Tropical, subtropical	Low	Mesophyles & Thermophyles	Arid to semi-arid	Rocky and saline soils, coastal (Lundgren & Christin, 2017; Lauterbach et al., 2019).
	Sedobassia	1	C_3-C_4 Intermediate	Annual	Temperate	Low	Mesophyles	Arid to semi-humid	Saline, arid environments, such as salt flats, saline lakes, and clay-rich soils (Shuyskaya et al., 2019).
Asteraceae	*Flaveria*	9	C_3-C_4 Intermediate	Annual, biennial, or perennial	Tropical, subtropical	Low–Med	Mesophyles	Arid to humid	Various habitats; primarily found in disturbed, sandy soils with some in tropical habitats (Lundgren & Christin, 2017; Adachi, 2022).
	Parthenium	1	Stable State C_2	Annual	Tropical, subtropical		Mesophyles & Thermophyles	Arid to humid	Various habitats including highly disturbed, sandy soils (Kore and Patil, 1995).
Boraginaceae	*Euploca*	5	C_3-C_4 Intermediate	Perennial	Tropical, subtropical	Low–High	Mesophyles & Thermophyles	Arid to humid	Arid to tropical habitats often in alkaline soils (Frohlich et al., 2022).
	Heliotropium	2	C_3-C_4 Intermediate	Annual	Tropical, subtropical, temperate	Low–High	Mesophyles	Arid to humid	Arid regions of Central and North America in disturbed and scrubland habitats with sandy soils (Vogan, Frohlich, & RF Sage, 2007; Muhaidat et al., 2011).
Brassicaceae	*Brassica*	1	Stable State C_2	Biennial or Perennial	Subtropical, temperate	Low	Mesophyles	Semiarid to humid	Mediterranean region from Italy to Northern Africa (Ueno, 2011; GBIF, 2024).
	Diplotaxis	3	Stable State C_2	Biennial	Tropical, subtropical, temperate	Low	Mesophyles	Arid to humid	Various habitats including disturbed soils (Lundgren & Christin, 2017).
	Hirschfeldia	1	Stable State C_2	Annual	Tropical, subtropical, temperate	Low	Mesophyles	Arid to humid	Arid to temperate habitats with disturbed soils (Taylor et al., 2023; Schlüter et al., 2023).

Table 2. *Cont.*

	Lineage	**No. C_2 Species**	**Hypothesized C_2 Type**	**Life History**	**Climatic Zone**	**Alt**	**Optimal Growth Temperature**	**Moisture Preferences**	**Habitat Descriptions**
	Moricandia	5	Stable State C_2	Perennial	Tropical, subtropical	Low	Mesophyles & Thermophyles	Arid to humid	Disturbed soils such as roadsides (McVetty, Austin, & Morgan, 1989; Perfectti et al., 2017).
Cleomaceae	*Cleome*	1	C_3-C_4 Intermediate	Annual	Tropical	Low–Med	Mesophyles & Thermophyles	Arid	Arid habitats with rocky soils (Feodorova et al., 2010).
Euphorbiaceae	*Euphorbia*	3	C_3-C_4 Intermediate	Perennial (sometimes woody)	Subtropical	Low	Mesophyles	Semiarid to humid	Disturbed arid regions with sandy and rocky soils (TL Sage et al., 2011).
Molluginaceae	*Hypertelis/ Paramollugo*	2	Stable State C_2	Annual	Tropical, subtropical	Low	Mesophyles & Thermophyles	Arid	Mainly in arid scrublands (Christin et al., 2011; Lundgren & Christin, 2017).
	Mollugo	1	C_3-C_4 Intermediate	Annual	Tropical, subtropical, temperate	Low	Mesophyles	Semiarid to humid	Dry, disturbed, sandy soils (Christin et al., 2011).
Portulacaceae	*Portulaca*	3	C_3-C_4 Intermediate	Annual	Tropical, subtropical	Low–Med	Psychrophiles & Mesophyles	Semiarid to humid	Warm habitats with varying rainfall (Voznesenskaya et al., 2017; Lundgren & Christin, 2017).
Scrophulariaceae	*Anticharis*	2	C_3-C_4 Intermediate	Annual	Subtropical	Low	Mesophyles	Arid to semi-arid	Arid and semi-arid regions with sandy soils (Khoshravesh et al., 2012).
Tribuloideae	*Tribulus*	1	C_3-C_4 Intermediate	Annual	Subtropical	Low	Mesophyles	Arid to semi-humid	Desert and shrubland in sandy soils (Prokofieva et al., 2022).
MONOCOTS									
Cyperaceae	*Eleocharis*	6	C_3-C_4 Intermediate	Perennial	Tropical, subtropical, temperate	Low–Med	Mesophyles & Thermophyles	Arid to humid	Wetlands with fluctuating water levels (Lundgren & Christin, 2017).
Poaceae	*Alloteropsis*	1	C_3-C_4 Intermediate; C_3 × C_4 hybrid	Perennial	Tropical	Low–Med	Mesophyles & Thermophyles	Humid	Shady, miombo woodlands (Lundgren et al., 2015; 2016).
	Homolepis	2	C_3-C_4 Intermediate	Perennial	Tropical	Low–Med	Mesophyles	Humid	Various habitats including disturbed habitats, grasslands, riversides, and pastures (Khoshravesh et al., 2016).
	Neurachne	1	C_3-C_4 Intermediate	Perennial	Subtropical	Low	Mesophyles & Thermophyles	Semi-arid to humid	Predominantly deciduous woodlands and scrubland with sandy to stony soils (Prendergast and Hattersley, 1985).
	Steinchisma	5	Stable State C_2	Perennial	Tropical, subtropical	Low	Mesophyles	Semi-humid to humid	Various habitats including disturbed grasslands, riversides, and wetlands (Edwards and Ku, 1987; Lundgren & Christin, 2017).

The photosynthetically diverse eudicot genus *Flaveria* (Asteraceae) is another excellent system to study evolutionary transitions across the C_3 to C_4 continuum. Zhao et al., (2022) show that stomatal traits gradually shift along the intermediate continuum of C_4 evolution in *Flaveria*. Specifically, they show that stomata density decreased, and stomatal length increased, stepwise from C_3 to C_2 to C_4 congeners, suggesting that the C_2 phenotype creates an intermediate stomatal phenotype across the C_3 to C_4 continuum in this eudicot lineage. A study distinguishing the direct (i.e., light-induced) from indirect (i.e., intercellular CO_2 concentration–induced) drivers of stomatal conductance found that the evolution of C_4 photosynthesis in *Flaveria* was accompanied by changes in the way that stomata sense light and leaf internal CO_2 concentrations, with C_2 *Flaveria* species again creating intermediate phenotypes between C_3 and C_4 congeners (Huxman and Monson 2003).

More research is needed to characterise broad trends in C_2 plant stomatal patterning. The literature has so far focused on the role of C_2 plants in facilitating evolutionary shifts in stomatal patterning across the C_3 to C_4 continuum, with little to no research available on stable state C_2 lineages or $C_3 \times C_4$ hybrid C_2 species. At this point, the research suggests that patterns of stomatal trait shifts across the C_3 to C_4 evolutionary continuums are lineage specific, however, the C_2 phenotype seems to largely function as an evolutionary intermediate step between the C_3 and C_4 extremes in these C_3-C_4 intermediate C_2 lineages.

4. Coordination between Stomatal and Minor Vein Densities in C_2 Leaves

Leaf stomata and vasculature networks function in concert to maintain homeostasis within the leaf and, as such, stomatal and vein development are often inherently linked (Fiorin, Brodribb, & Anfodillo, 2016, Zhao et al., 2017). In monocot leaves, which generally have parallel venation, stomata tend to be linearly distributed along veins, such that stomatal density is positively correlated with vein density, and often minor vein density specifically (Rudall, Chen, & Cullen, 2017; Zhang et al., 2022). In eudicot leaves, however, stomatal spatial patterning is seemingly less ordered, with broadleaved plants possessing diverse, net-like vasculature systems (Figure 1B; Conklin, Strable, & Scanlon, 2019). Despite the scattered arrangement of stomata in eudicot broadleaf species, stomatal development is still tightly and positively correlated with pavement cell number consistently across multiple lineages (Conklin, Strable, & Scanlon, 2019; Zhang et al., 2022).

The coordination between stomatal and venation development may decouple over the evolutionary continuum between C_3 to C_4 phenotypes. Indeed, C_4 species are usually associated with having higher vein density (or smaller interveinal distances) than C_3 species (e.g., Ueno et al., 2006; Lundgren, Osborne, & Christin, 2014; Lundgren et al., 2019) yet, as reported above, they often have lower stomatal densities than C_3 species. The enhanced water use efficiency of C_4 biochemistry may uncouple the strict stomatal—vasculature relationship seen in C_3 species. In addition, this discrepancy may arise as a result of differing points along the C_4 evolutionary trajectory that venation increases in each independent emergence. Increasing vein density (or decreasing interveinal distances) is a precursor to C_4 emergence, occurring within C_3 lineages and acting as an anatomical enable of C_4 evolution (RF Sage 2004, Christin et al., 2013, Griffith et al., 2013). Perhaps increases to both vein and stomata densities occurred within C_3 lineages prior to C_4 emergence, while subsequent changes to vein density that occur within the C_3-C_4 intermediate stages of C_4 evolution are then decoupled from the tight stomata—vasculature relationship due to increased efficiencies of the CCM biochemistries. Indeed, vein density has been reported to be higher in C_2 species compared to close relatives that use C_3 photosynthesis in the eudicot lineages *Blepharis* (Stata 2023), *Cleome* (Marshall et al., 2007), *Diplotaxis* (Ueno et al., 2006), *Flaveria* (McKown and Dengler 2007), *Hypertelis* (Christin et al., 2011), *Mollugo* (Christin et al., 2011), and *Salsola* (Voznesenskaya et al., 2013) and the monocot lineages *Alloteropsis* (Bianconi et al., 2022), *Homolepsis* (Christin et al., 2011; Alvarenga et al., 2025), *Neurachne* (Khoshravesh et al., 2020) and *Steinchisma* (Khosravesh et al., 2016a). However, the C_2 phenotype does not always enhance vein density, with C_2 species in the eudicot lineages *Anticharis* (Khoshravesh et al., 2012), *Heliotropium* (Vogan, Frohlich, & RF Sage, 2007), *Portulaca* (Voznesenskaya et al., 2017), *Moricandia* (Schlṳter et al., 2017), and *Euphorbia* (TL Sage et al., 2011) reporting similar or lower vein density compared to their C_3 close relatives. Moreover, shifts in vein density may occur for other reasons linked to CCM functionality, such as improving connectivity between mesophyll and bundle sheath cells, or reasons not directly related to CCMs, such as selection pressure to improve light capture or leaf hydraulic conductance (Ogle 2003; Lundgren et al., 2014; Gao et al., 2023).

Linkages between stomatal and venation traits along the C_3 to C_4 continuum seem to be lineage-specific and may be at odds with maintaining the positive stomata-venation relationship along the C_3-C_4 continuum, which could have implications for engineering a stomatal trait-related C_2 phenotype into C_3 plants. Such stomata-venation relationships have been hypothesised in a ‘broken stick’ model between C_3, C_2, and C_4 relatives, based on studies from eudicots and monocots, wherein stomatal and anatomical traits may reach a maximum at different points along the photosynthetic continuum (Zhang et al., 2022; Stata, 2023). Moreover, C_3 species from arid zones that are closely related to C_2 species are noted to have increased vein density due to evolved decreases in diffusion distance of metabolites and gases particularly between mesophyll and bundle sheath tissues (Roth-Nebelsick et al., 2001). Therefore, such increases in vein density along the photosynthetic continuum may be masked by ecological history (Muhaidat et al., 2011;

Taylor et al., 2010). Declines in stomatal density (as mentioned above), and thus stomatal development, from C_4-like to C_4 *Flaveria* species may result from constraints in vein development, but this may not be the case in earlier stages of the photosynthetic continuum (i.e., between C_3 to C_2 type I). Therefore, shifts in stomatal and venation traits appear to arise late along the C_3 to C_4 continuum, potentially peaking in C_4-like phenotypes, meaning that high foliar vein density may be a prerequisite for C_4 evolution (Christin et al., 2013; Griffiths et al., 2013; Lundgren et al., 2019a).

5. Shifting the Mesophyll: Bundle Sheath Area Ratio

The distinct Kranz leaf anatomy of C_4 plants has been known for the last 150 years (Duval-Jouve, 1875; Haberlandt, 1884; but see Voznesenskaya et al., 2001), however, C_2 leaf anatomy remains less clear. While mesophyll tissue is the primary site of photosynthetic activity in C_3 plants, the bundle sheath becomes increasingly important to photosynthesis functionality with the introduction of C_2 and C_4 CCMs. As a result, the ratio of mesophyll to bundle sheath tissue areas (M:BS) shrinks along the C_3 to C_4 continuum in eudicots (Muhaidat, RF Sage, & Dengler, 2007; 2011; Voznesenskaya et al.; 2013; Yorimitsu et al., 2019; Leung et al., 2024) and grasses (Hattersley 1984; Dengler et al., 1994; Lundgren et al., 2019a). With each independent emergence of photosynthetic innovation, these shifts in M:BS can be achieved via any combination of underlying traits including the size, number, and distribution of each cell type (reviewed in Lundgren et al., 2014). The drivers of M:BS ratio shifts can be caused by different underlying traits in C_2 versus C_4 species within the same lineage. In the grass *Alloteropsis semialata*, for example, lower M:BS ratios are achieved in C_2 accessions via a reduction in mesophyll tissue area through the development of fewer mesophyll cells, while lower M:BS ratios are achieved in C_4 accessions by increasing vein density, specifically via the proliferation of minor veins (Lundgren et al., 2019a).

M:BS ratios in the C_2 grasses *Homolepis aturensis* and *Steinchisma hians* were recorded to be more than 2.5 time smaller than their C_3 close relatives and statistically similar to that recorded in their C_4 relatives (Khoshravesh et al., 2016). A recent study by Alvarenga et al., (2025) on the grass subtribe Arthropogoninae, found that both C_2 (*Homolepis longispicula* and *Homolepis aturensis*) and C_4 (*Mesosetum loliiforme*) close relatives presented with lower M:BS ratios compared to C_3 relatives via higher vein density, with minor vein proliferation playing an important role in increasing leaf bundle sheath volume the C_4 phenotype. In the eudicot *Flaveria* lineage, lower M:BS was also achieved in the C_4 *Flaveria bidentis* via increased vein density through minor vein proliferation compared to C_3 *Flaveria robusta*, as well as through the development of fewer and smaller mesophyll cells (McKown and Dengler 2009). In this lineage, C_2 *Flaveria* functionally lower M:BS by increasing bundle sheath cell size and reducing mesophyll tissue areas, specifically by reducing mesophyll tissue layers (McKown & Dengler 2007). In the C_2 eudicot *Euphorbia acuta*, M:BS is less than half that measured in the C_3 congener *Euphorbia angusta* and similar to its C_4 congener *Euphorbia lata* (TL Sage et al., 2011). This lower M:BS was achieved in *E. acuta* via decreases in mesophyll tissue (via smaller mesophyll cell size and thinner leaves, despite larger interveinal distances) and increases in bundle sheath tissue via enlargement of bundle sheath cells. Thus, in this *Euphorbia* lineage, the C_2 phenotype is intermediate between that of C_3 and C_4 congeners. Research on C_2 leaf anatomy consistently points to an important role for the C_2 phenotyping in shifting the M:BS ratio, which is achieved by unique combinations of underlying traits in each lineage.

6. Structure–Function Relationships in C_2 Leaves

Gaseous diffusion through leaf tissues is influenced by both stomatal patterning and leaf and tissue structure (Terashima et al., 2011). Stomatal density and size are positively correlated with maximal rate of diffusion of carbon dioxide through the stomata to the mesophyll layer (Harrison et al., 2019). Stomatal density is also genetically modulated by other leaf anatomical traits, such as mesophyll area and mesophyll air space patterning (Lundgren et al., 2019b; Baillie and Fleming, 2020), further linking leaf and mesophyll anatomies in optimising gas exchange (Lehmeier et al., 2017). Spongy mesophyll cellular arrangement and air space patterning influence CO_2 diffusion between mesophyll and intracellular spaces (mesophyll conductance, g_m) (Evans, 2021). Therefore, low stomatal and mesophyll conductance limit CO_2 fixation and ultimately decrease photosynthetic nitrogen and intrinsic water use efficiencies within C_3 species. However, understanding the importance of mesophyll conductance in gas exchange efficiencies across the C_3 to C_4 phenotypic continuum remains challenging due to uncertainties in quantification and modelling of anatomical traits, such as mesophyll cellular, airspace, and conductance patterning, apoplast thickness and porosity, and ultrastructure (Lundgren & Fleming, 2020) across the C_3 to C_4 continuum.

Leaf-level water use efficiency reflects the balance between net carbon assimilation and water loss from the leaf. C_4 leaves consistently present with higher water use efficiency than C_3 leaves under environments that promote high rates of photorespiration (RF Sage, 2004; Osborne & Sack, 2012). This generally results from the efficient C_4 biochemistry facilitating a combination of higher rates of photosynthesis at lower stomatal conductance than C_3 species. How the leaf structure—function relationships shift along the C_3 to C_4 evolutionary continuum to influence physiological efficiencies remains unclear. One reason that leaf structure—function patterns are unclear in C_2 species is their physiological plasticity. At low rates of photorespiration, C_2 plants should function like C_3 plants, conducting the Calvin-Benson-Bassham cycle within the mesophyll. However, when environmental conditions shift to promote higher rates of photorespiration, C_2 plants initiate their glycine shuttle which allows them to function more efficiency, not too unlike a C_4 species. Therefore, leaf level physiology differs strongly in C_2 plants depending on the environment, rates of photorespiration, and consequent efficacy of the glycine shuttle. Many studies have recorded higher rates of net carbon assimilation in C_2 species compared to C_3 relatives across diverse genera including, for example, *Alternanthera* (Rajendrudu, Prasad, & Das, 1986), *Diplotaxis* (Ueno et al., 2006), *Heliotropium* (Vogan, Frohlich, & RF Sage, 2007), *Mollugo* (Kennedy, Eastburn, & Jensen, 1980), and *Flaveria* (Monson, 1989; Zhao et al., 2022). While this is an intriguing trend, net carbon assimilation does not present as a consistent phenotype across C_2 species and heavily depends on experimental growth and measurement conditions, and associated rates of photorespiration during measurements. With that said, water use efficiency is often higher in C_2 eudicot species than their close C_3 relatives across diverge lineages, including *Alternantheara* (Rajendrudu, Prasad, & Das, 1986), *Blepharis* (Stata, 2023), *Cleome* (Voznesenskaya et al., 2007), *Diplotaxis* (Ueno et al., 2006), *Euphorbia* (TL Sage et al., 2011), and *Heliotropium* (Vogan, Frohlich, & RF Sage, 2007). In monocot lineages, however, C_2 plants consistently present similar water use efficiencies as their C_3 relatives (Bianconi et al., 2022; Khoshravesh et al., 2016; 2020). Improvements to water use efficiencies may therefore occur later along the C_3 to C_4 continuum in monocot than eudicot lineages. Nevertheless, the high physiological plasticity observed in C_2 species, regardless of lineage, favours their persistence and ecological success across a wide range of environments.

7. Ecological and Geographical Distributions of C_2 Plants

C_2 plants are broadly distributed across the Earth's surface. Largely centred in tropical, sub-tropical, and temperate latitudes between 46° S and 66° N, they inhabit every continent except Antarctica (Table 1; Figure 3). They exist broadly across elevations, from sea level to high altitudes (~4900 feet above sea level; Table 1), but primarily inhabit low altitudes. C_2 species prefer moderate temperatures and thrive between 10 °C and 30 °C growing season temperature (i.e., mesophyles), however, some C_2 species can also often thrive at very high temperatures (i.e., thermophyles) (Table 1). C_2 species inhabit broad precipitation gradients spanning tropical, sub-tropical, temperate, and arid regions (Figure 3, Table 1). C_2 monocot species are commonly found in wetlands, deciduous forests, grasslands, and scrubland in disturbed areas, while C_2 eudicot species are commonly described to inhabit dry, disturbed habitats with sandy and rocky soils (Table 1). Because many C_2 species have broad environmental tolerance and thrive in disturbed areas, they often become weedy. Indeed, many C_2 species are common global weeds, including *Parthenium hysterophorus*, *Chenopodium album*, *Mollugo verticillata*, and *Eleocharis atropurpurea* (Global Invasive Species Database, 2024; Irakiza et al., 2021; Shabbir et al., 2024). These weedy species likely largely drive the broad patterns of global ecological and geographical distributions (Lundgren & Christin, 2017). Indeed, some C_2 species have very narrow ecological niches and geographical ranges. For example, C_2 accessions of the grass *Alloteropsis semialata* are confined to wooded savannas in central eastern Africa (Lundgren et al., 2015; Bianconi et al., 2022).

Phenotypic plasticity, or the ability of a genotype to express different phenotypes in response to environmental conditions (Pigliucci, 2001), can be one factor in plant species' distributions across ecological and geographical spaces. It has been postulated that C_4 species are more canalised, and less phenotypically plastic, than C_3 species which may explain their generally narrower ecological niche space and geographical distributions compared with C_3 relatives (RF Sage and McKown, 2006). The physiological plasticity present in C_2 species, as described above, may partially explain their remarkably large geographical and ecological distribution. Narrowly distributed C_2 species may instead be canalized into troughs within the evolutionary landscape, limiting their ecological and geographical spread.

8. Concluding Remarks

This review discusses C_2 photosynthesis across scales, drawing links between biochemistry and leaf structure and ultimately to ecological and geographical breadth. We highlight where broad trends across C_2 species are evident, while also identifying key research gaps that remain. For example, further studies are required to interrogate leaf anatomy in depth, including how mesophyll organisation influences gas exchange in C_2 leaves. Research should also focus on agriculturally important plant families, to better understand the C_2 phenotypes within them and identify potential opportunities for crop improvement via C_2 photosynthesis engineering programs. Engineering C_2 photosynthesis into C_3 crops, however, will inherently need to be bespoke and/or lineage specific, as this review shows, with monocots and eudicot species presenting distinct developmental challenges to incorporate C_2 traits. Overall, the C_2 CCM offers valuable opportunities to potentially improve C_3 crops under climate change-related stresses such as high temperatures and drought, and climatic instability, to contribute to ongoing efforts to achieve future food security at the global scale. However, discussions of C_2 species in the literature currently lump C_3-C_4 evolutionary intermediates, stable state C_2 species, and $C_3 \times C_4$ hybrid species together which may mask important and useful diversity distinguishing these types. We urge that these types should be distinguished, when possible, and researched independently. These different origins of C_2 species likely explain the broad diversity of phenotypes and biogeography observed across C_2 species and may provide important insights for crop improvement.

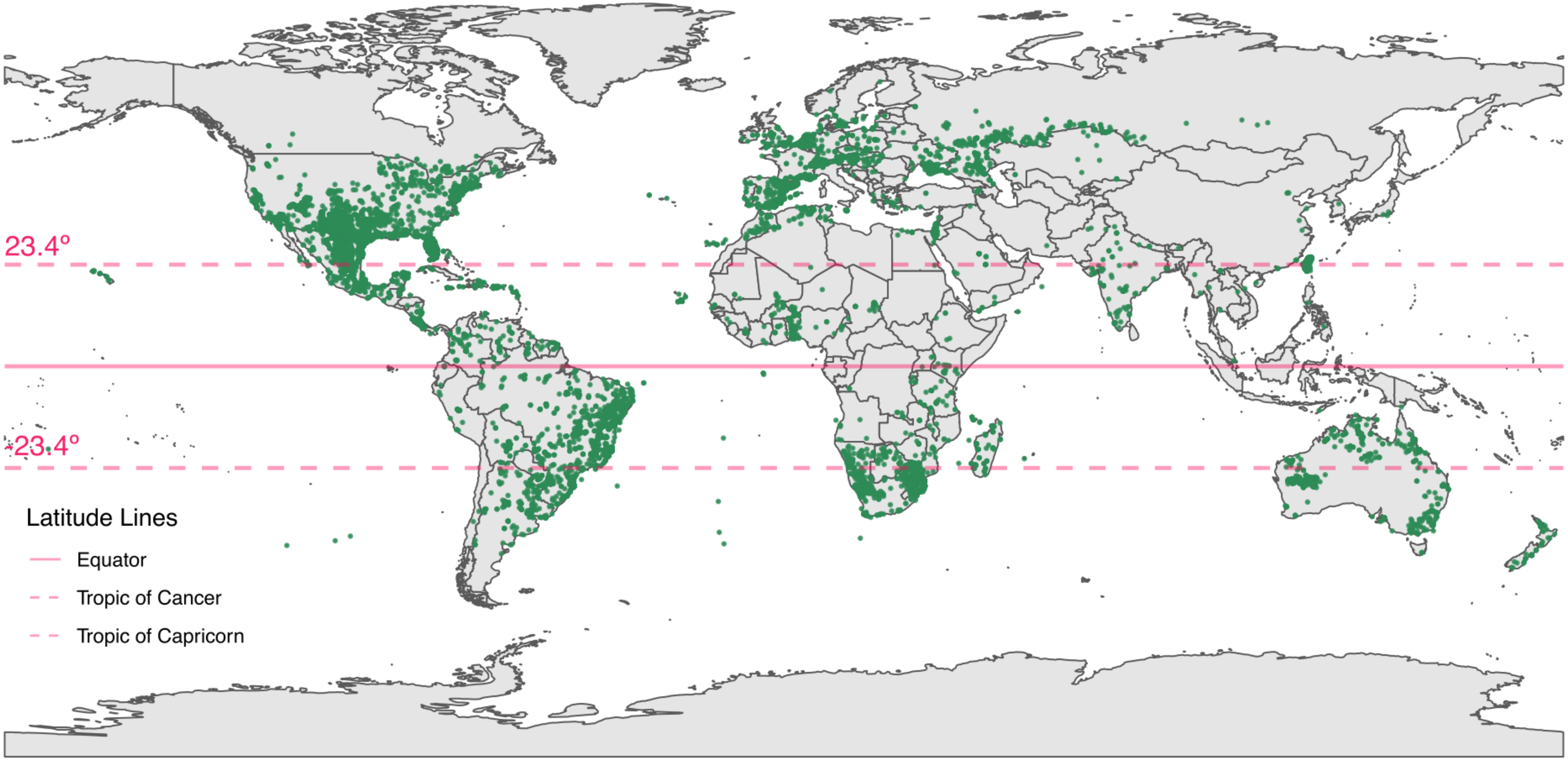

Figure 3. Global distributions of C_2 species. Geographical distributions of C_2 species listed in Table 1, based on occurrence data available at the Global Biodiversity Information Facility (GBIF.org; accessed 21 August 2024). Occurrence data were extracted using the rgbif package (Chamberlain et al., 2023) and cleaned following general GBIF guidelines (GBIF, 2022). These included excluding data associated with zoos, botanical gardens, arboreta, or GBIF headquarters (based on the institutionCode field), located at coordinates (0,0), or flagged with known issues such as ZERO COORDINATE, COUNTRY COORDINATE MISMATCH, or GEODETIC DATUM INVALID. Duplicate entries were also omitted using the *occurrenceID* field. The cleaned dataset was converted into a spatial features (sf) object for mapping and climate data extraction. Basemap layers were added with *rnaturalearth* (Massicotte & South, 2023) and bioclimatic variables were extracted from WorldClim v2 (Fick & Hijmans, 2017) using *terra* and *geodata* R packages (Hijmans, 2023; Hijmans et al., 2024).

Author Contributions

HRR and MRL conceived the review. HRR, CFC, and ABC prepared figures and tables, and contributed ideas. All authors wrote the manuscript.

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No novel data were generated in this manuscript.

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The authors declare no conflict of interest

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