

LEAF ANATOMY CHARACTERIZATION OF FOUR *Apochloa* SPECIES: A C3 GENUS RELATED TO EVOLUTION OF C4 PATHWAY IN GRASSES

Caracterización anatómica de hojas de cuatro especies de *Apochloa*: un género relacionado con la evolución de la vía C4 en gramíneas

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Received: 29th October 2019, Returned for revision: 14th April 2019, Accepted: 23th June 2020.

Associate Editor: Susana Feldman.

Citation/Citar este artículo como: Mendonça AMC, Viana PL, Barbosa JPRAD. Leaf anatomy characterization of four *Apochloa* species: a C3 genus related to evolution of C4 pathway in grasses. Acta Biol Colomb. 2021;26(1):12-18. Doi: <http://dx.doi.org/10.15446/abc.v26n1.83228>

ABSTRACT

Leaf anatomy characteristics provide important evidences about the transition between C3 and C4 pathways. The C4 photosynthesis pathway allowed to reduce the C3 photorespiratory rate, concentrating CO₂ around the Rubisco site and using structures and machinery already presented in C3 plants. In monocots, it is observed a high number of C4 lineages, most of them phylogenetically related to C3 groups. The genus *Apochloa* (C3), subtribe Arthropogoninae, is related to two C4 genera *Coleataenia* and *Cyphonanthus*. The aim of this study was to evaluate four *Apochloa* species in order to establish anatomical characteristics related to the evolution of C4 pathway in this group. By means of transverse sections fully expanded leaves of *A. euprepes*, *A. lorea*, *A. molinooides*, and *A. poliophylla* were collected and the characteristics of the mesophyll (M) and bundle sheath (BS) cells were determined. These species showed a rustic Kranz anatomy with enlarged and radial arranged BS cells, which have few organelles organized in a centrifugal position. Although the modifications of BS cells are probably related to the maintenance of plant water status, we also discuss the evolution for the establishment of C4 photosynthesis in the related C4 genera.

Keywords: *Apochloa* spp, C4 photosynthesis, leaf anatomy, poaceae, subtribe arthropogoninae.

RESUMEN

Las características de la anatomía de la hoja proporcionan evidencias importantes sobre la transición entre las vías C3 y C4. La fotosíntesis C4 surgió para reducir la tasa de fotorrespiración C3, concentrando el CO₂ alrededor del sitio de la Rubisco y utilizando estructuras y maquinaria ya presentes en las plantas C3. En monocotiledóneas, se observa un alto número de linajes C4, la mayoría de ellas filogenéticamente relacionadas con grupos C3. El género C3 *Apochloa*, que pertenece a la subtribu Arthropogoninae, está relacionado con dos géneros C4 *Coleataenia* and *Cyphonanthus*. En este contexto, el objetivo fue evaluar cuatro especies de *Apochloa* para establecer las características anatómicas relacionadas con la evolución de la vía C4 en este grupo. Se colectaron hojas completamente expandidas de *A. euprepes*, *A. lorea*, *A. molinooides* y *A. poliophylla* y se determinaron las características de las células del mesófilo (M) y del haz de la vaina (HV) a partir de secciones transversales de la hoja. Las especies presentaron una anatomía rústica de Kranz con células HV agrandadas y de distribución radial, con pocas organelas organizadas en posición centrífuga. Aunque las modificaciones de las células HV están probablemente relacionadas con el mantenimiento del estado hídrico de la planta, se puede inferir que facilitan el establecimiento de la fotosíntesis en los géneros C4 relacionados.

Palabras clave: anatomía de la hoja, *Apochloa* spp. fotosíntesis C4, poaceae, subtribu arthropogoninae.

INTRODUCTION

Leaf anatomy provides important information about aspects of plant physiology. Thus, anatomy techniques are important tools to link morphological and physiological traits of plants (Brown, 1958; Hattersley and Watson, 1976). In studies about transition between the C3 and C4 pathways, leaf anatomy characterization is the first step in order to determine transverse sectional characteristics of the mesophyll (M) and the bundle sheath (BS) cells, and the degree of approximation between these two compartments (Dengler *et al.*, 1994; Khoshravesh *et al.*, 2016; Lundgren *et al.*, 2019). The C4 via of the photosynthesis is an evolutionary novelty derived from C3 pathway, in response to the increase of photorespiratory activity (Sage, 2016). As most of the C4 plants have a spatial separation, where the initial CO₂ fixation occurs in M cell and the decarboxylation in the BS cell, there is a CO₂ concentration mechanism around the Rubisco site, avoiding its oxygenase activity (Sage *et al.*, 2011; Sage *et al.*, 2014).

It is well documented that first steps for C4 evolution includes anatomical and ultrastructural modifications to establish the Kranz anatomy (Gowik *et al.*, 2011). Thus, a reduction in the distance between two vascular bundles for the metabolite shuttle and an increase in the number of organelles in the bundle sheath cells for the photosynthetic activity are expected (Lundgren *et al.*, 2019). Interestingly, some C3 species have a leaf anatomy closely related to C4 plants, especially in Poaceae family (Lundgren *et al.*, 2014). In these cases, a large investment in BS tissue, which requires just few modifications in order to set C4 pathway, is observed (Christin *et al.*, 2013; Lundgren *et al.*, 2014).

During the evolution of photosynthesis, C4 pathway has evolved independently more than 60 times in angiosperms (Sage, 2016). Monocots account the highest number of C4 species (Sage *et al.*, 2011), especially in the Poaceae family, which has the largest number of C4 transitions, about 20 times (Christin *et al.*, 2012). All C4 grasses are clustered in PACMAD clade (Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae, and Danthonioideae), showing that this clade probable has some characteristic to facilitate this evolution (Christin *et al.*, 2010; GPWG II, 2012). Of all C4 origins in PACMAD, 15 occurred in the subfamily Panicoideae, its greatest lineage, which includes the subtribe Arthropogoninae (Giussani *et al.*, 2001; Edwards and Smith, 2010; GPWG II, 2012).

The subtribe Arthropogoninae has seven genera with C3 photosynthesis and nine genera with C4 photosynthesis, type NADP-ME (Morrone *et al.*, 2012). Among them, the genus *Apochloa* Zuloaga & Morrone is described as a C3 one, and it is phylogenetically related with two C4 genera: *Coleataenia* Griseb. and *Cyphonanthus* Zuloaga & Morrone (GPWG II, 2012; Morrone *et al.*, 2012). Considering the modifications that occur in C3 grasses related to C4 genera, it is important to determine whether *Apochloa* species present

anatomical traits that can be considered a pre-disposition to the C4 pathway. Thus, the aim of this paper was to describe leaf anatomy by means of transverse sections of four *Apochloa* species in order to investigate if the genus has characteristics that facilitate the C4 evolution in the subtribe Arthropogoninae.

MATERIAL AND METHODS

Site description and plant material

Anatomical studies were carried out with four *Apochloa* species: *A. euprepes* (Renvoize) Zuloaga & Morrone, *A. lorea* (Trin.) Zuloaga & Morrone, *A. molinooides* (Trin.) Zuloaga & Morrone, and *A. poliophylla* (Renvoize & Zuloaga) Zuloaga & Morrone. Species were collected in February 2016, at the Serra do Cipó, Santana do Riacho county, Minas Gerais state, Brazil (19° 28' S and 43° 58' W). This area is localized in the southern region of the Espinhaço mountain chain and it is characterized by rupestrian fields (Medina *et al.*, 2007). The weather of the region is classified as CWB of Köppen (1948), altitudinal tropical, with warm and rainy summers and a dry season that can last seven months (Ribeiro *et al.*, 2009). A soil sample where the species were found was collected. Soil pH was measured in solution using a 1:2.5 soil/ water ratio (DM-22 Digimed pHmeter); sand, silt, and clay contents (dag kg⁻¹) and organic material (dag kg⁻¹) were determined using the pipette method. The region was characterized by acid and sandy soil, with low content of organic material (Table 1).

Leaf anatomy

Healthy, turgid, and fully expanded leaves from three individuals of each species, situated at minimum distance of 1 m among the plants were harvested. This guarantees that the material belongs to different individuals.

Fragments of 2 cm² from the centre region of the leaves were fixed in 1 % (v/v) paraformaldehyde and 1 % (v/v) glutaraldehyde in 0.05 M sodium cacodylate buffer, at

Table 1. Soil characteristic of the site of collection (Serra do Cipó, Santana do Riacho county, Minas Gerais state, Brazil) of *Apochloa* species.

Soil characteristics	
pH	4.5
Organic Material (dag kg ⁻¹)	1.50
Soil characterization	Sandy texture
Sand	59
Silt (dag kg ⁻¹)	39
Clay	2

9-11 am. The tissue was dehydrated through a graded series of ethanol and embedded in LR White resin (Voznesenskaya *et al.*, 2013). The leaf anatomy was studied by transverse sections (2-3 μm) obtained using a rotative microtome (model MRP-09 LUPETEC) and stained with 1 % toluidine blue.

A total of 36 observations per species were analyzed: three different sections and four images per sections for each specimen. The distance (μm) and the number of cells between two veins, percentage of M and BS cells, and M:BS ratio were determined. The percentage of M and BS cells was calculated using a stereological grid of 500 random points layered on transverse section images and counting the proportion of points in M and BS cells in comparison to the total points (Mckown and Dengler, 2007).

Statistical analyses

Leaf anatomy data met the assumptions of normality, homoscedasticity, and independence and then, were compared with one-way ANOVA followed by the Tukey's test ($p < 0.05$), using Sigma Plot version 11.0.

RESULTS

The leaf transverse sections showed that the four *Apochloa* species had uniseriate epidermis with bulliform cells with rounded shape in the adaxial epidermis. Projection of the sclerenchyma connects the vascular bundles to the adaxial

and abaxial epidermis. The mesophyll shows intercellular spaces and an arrangement of cells around the vascular bundles, with enlarged and uneven BS cells (Fig. 1).

All the species analyzed presented two layers of cells around the vascular bundle, being the inner layer of thick-walled cells characterized as mestome sheath and the outer layer as parenchymatous bundle sheath. In spite of the enlargement of BS cells, only few chloroplasts organized in a centrifugal position were observed, while in the M cells the organelles showed a periphery position pattern (Fig. 2).

Leaf anatomical traits showed significant difference between the species (Table 2). The highest distance between veins (519.0 μm) was observed for *A. molinioides*, while lower values were observed for *A. euprepes* and *A. poliophylla* (217.5 and 200.8 μm , respectively). In relation to the number of M cells between two veins, the lower values were also recorded for *A. poliophylla* and *A. euprepes*. Although these species had the lowest values of distance and number of cells between veins, and percentage of mesophyll cells, *A. euprepes* showed the highest percentage of BS cells (24 %), which determined more than 30 % decrease in the M:BS ratio. The enlargement of BS at the expense of M cells is a characteristic strongly related to the establishment of C4 pathway.

DISCUSSION

In all *Apochloa* species analyzed an enlargement of the bundle sheath (BS) cells was found, an anatomical leaf

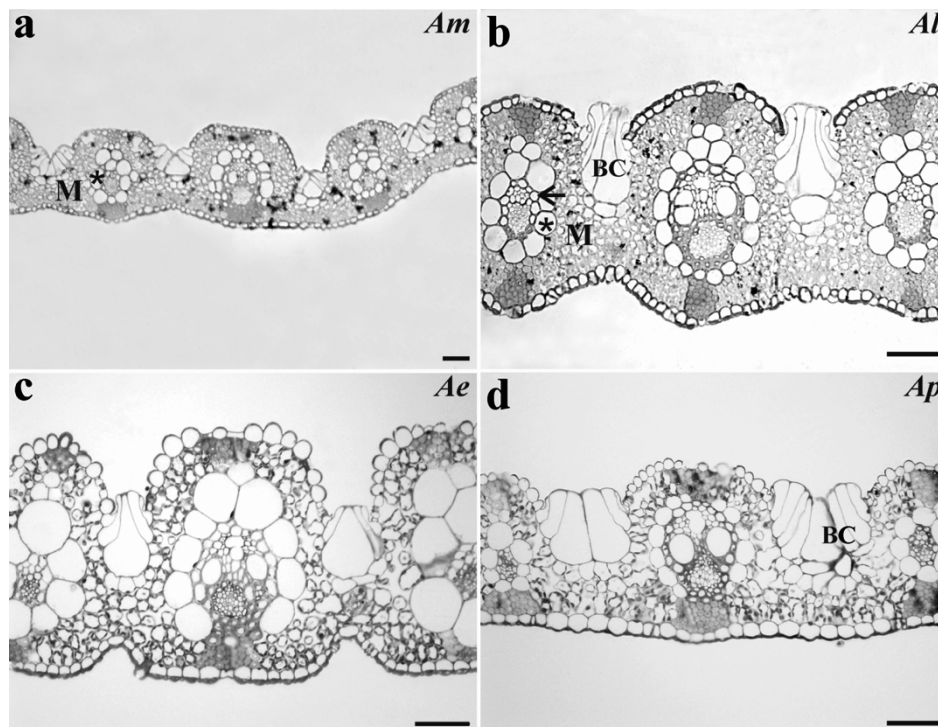


Figure 1. Leaf transverse sections from **a.** *Apochloa molinioides* (Am), **b.** *Apochloa lorea* (Al), **c.** *Apochloa euprepes* (Ae), and **d.** *Apochloa poliophylla* (Ap). M, mesophyll; asterisk, bundle sheath; BC, bulliform cell. Scale bar= 50 μm .

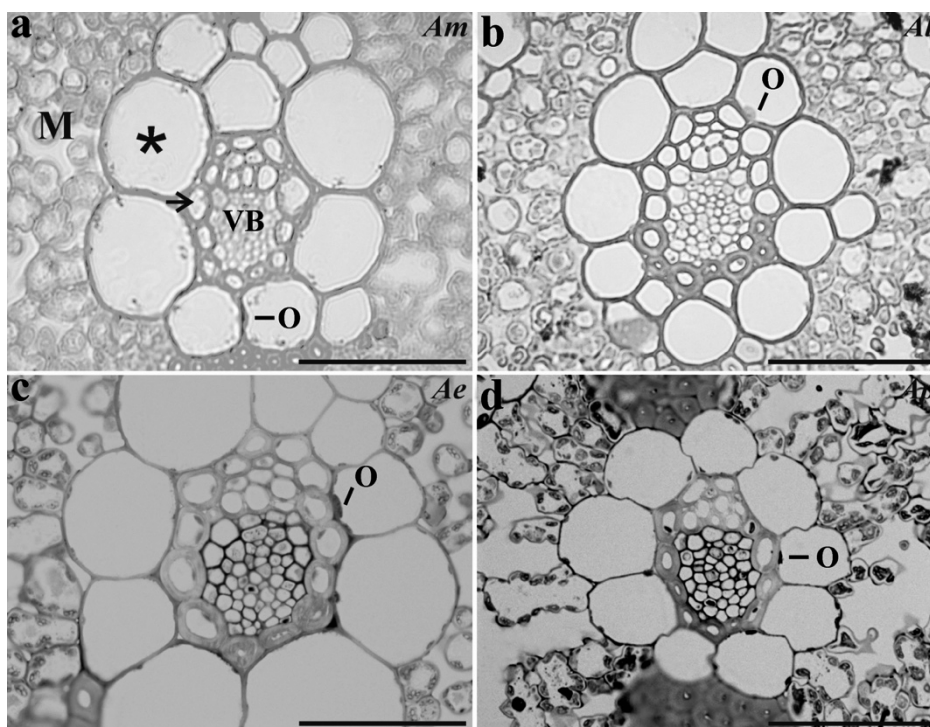


Figure 2. Bundle sheath cells organization from **a.** *Apochloa molinioides* (Am), **b.** *Apochloa lorea* (Al), **c.** *Apochloa euprepes* (Ae), and **d.** *Apochloa poliophylla* (Ap). M, mesophyll; asterisk, bundle sheath; arrow, mestome sheath; VB, vascular bundle; O, organelle. Scale bar= 50 μm .

trait commonly observed in intermediate species allowing to establish a C4 pathway (Gowik and Westhoff, 2011; Muhaidat *et al.*, 2011). In grasses, BS cells (parenchymatous cells) are organized in one or two layers, so that in the species with two layers, the inner layer called mestome sheath has small cells with suberized thick walls and absence of chloroplasts or other organelles, and it is located between the vascular tissue and the outer layer of parenchymatous bundle sheath (O'Brien and Kuo, 1975; Hattersley *et al.*, 1976; Dengler *et al.*, 1985; Lundgren *et al.*, 2014; Miyake, 2016), a typical organization observed in *Apochloa* species.

The emergence of Kranz anatomy is characterized by the presence of a concentric layer of cells around the vascular bundle, which allows the metabolic shuttle between M and BS cells, and in consequence the emergence of CO₂ concentration mechanism (Sage *et al.*, 2012; Lundgren *et al.*, 2014). Thus, the presence of enlarged and radial arranged

bundle sheath cells (parenchymatous cells) around the bundles can be considered a rustic Kranz anatomy (Muhaidat *et al.*, 2011); with the exception that they are not surrounded by a concentric layer of mesophyll cells (Rawsthorne, 1992) and a few intercellular spaces. This concentric organization is common in C3 grasses related to C4 groups of the family Poaceae (Lundgren *et al.*, 2014), as observed for *Apochloa* species. Besides that, the enlargement of the bundle sheath cells may represent an adaptation for C4 physiology, once this cell needs to accommodate more and larger chloroplasts to guarantee an efficient photosynthetic cycle (Lundgren *et al.*, 2019).

During the development of C4 pathway, the reduction in the distance and in the number of cells between two veins and the decrease of the M:BS ratio are common, which enables the metabolite shuttle between mesophyll and bundle sheath cell compartments (Lundgren *et al.*, 2014).

Table 2. Leaf anatomical characteristics of *Apochloa* species from Serra do Cipó, Santana do Riacho county, Minas Gerais state, Brazil.

Species	Distance between two veins (μm)	Number of M cells between two veins	Mesophyll (%)	Bundle Sheath (%)	M:BS ratio
<i>A. molinioides</i>	519,0 \pm 9,0 ^a	10,0 \pm 0,42 ^{ab}	28 \pm 1,1 ^c	14 \pm 0,3 ^b	2,1 \pm 0,08 ^b
<i>A. lorea</i>	335,0 \pm 3,4 ^b	10,0 \pm 0,48 ^b	30 \pm 0,5 ^b	14 \pm 0,4 ^b	2,3 \pm 0,06 ^{ab}
<i>A. euprepes</i>	217,5 \pm 6,8 ^c	6,8 \pm 0,20 ^a	33 \pm 0,5 ^{ab}	24 \pm 1,3 ^a	1,5 \pm 0,12 ^c
<i>A. poliophylla</i>	200,8 \pm 9,5 ^c	8,1 \pm 0,40 ^a	36 \pm 0,5 ^a	11 \pm 0,4 ^b	3,4 \pm 0,10 ^a

Values represent mean \pm SE, n= 36 observations per species. In each column, values followed by the same letter are not different (one-way ANOVA and the Tukey's test; p<0.05).

However, even with the reduction observed for the *Apochloa* species, our findings are still in the range of values found for C3 grasses (Christin *et al.*, 2013). The C3 grasses *Dicanthelium oligosanthes* (Schult.) Gould and *Panicum bisulcatum* Thunb. had distance between veins of 206 and 240 μm , respectively; figures more than 50 % higher than the studied C4 grasses (Khoshravesh *et al.*, 2016). High distance between veins was also observed for the C3 grass *Jansenella griffithiana* (Müll. Hal.) Bor, with a mean of 471 μm (Bianconi *et al.*, 2020). In relation to the number M cells between veins, C3 PACMAD species usually have three times more cells than C4 PACMAD ones (Ermakova *et al.*, 2020). This pattern was observed for the C3 and C4 grasses *Neurachne alopecuroidea* R. Br. and *Neurachne minor* S. T. Blake, with 6.5 and four cells, respectively (Khoshravesh *et al.*, 2020).

In fact, previous studies already pointed out that the genus *Apochloa* does not present specialized chloroplasts in the bundle sheath cells (Zuloaga *et al.*, 2010), as it was observed in all four species analyzed here. The ability to fix and reduce CO_2 in the BS cells is another step of BS cell activation and participation in the photosynthesis process (Gowik and Westhoff, 2011; Sage *et al.*, 2014). In order to confirm the presence of photosynthetic enzymes in cells, studies of ultrastructure and enzymatic immunolocalization are needed. However, scarce organelles, mainly chloroplasts, found in these parenchymatous cells may indicate that the engagement in carboxylation activity of photosynthesis is not the main function.

In this case, the enlarged BS cells may have another function, related to the environmental conditions in the area of collection. Sandy soils, poor in organic material, have a low capacity of water retention. Thus, the weather and soil characteristics provide low water availability for the most part of the year, even during the rainy season (Giulietti *et al.*, 1987; Castro and Menezes, 1995). In fact, bundle sheath cells of C3 tropical grasses have functions related to water supply (Sage, 2001; Griffiths *et al.*, 2013). It is related that C3 PACMAD grasses have a high tolerance to the increase of evaporative demand through the investment in bigger BS cells (Griffiths *et al.*, 2013). Under high temperature or low air humidity, and low water availability bundle sheath cells may store and regulate the water flux to the mesophyll, maintaining the leaf hydraulic integrity (Griffiths *et al.*, 2013), besides preventing air entering the xylem (Sage, 2001; Leegood, 2008). In this context, the water reserve function of BS cells has been considered a predisposition for C4 evolution in grasses, favouring the occupation of tropical and subtropical environments (Sage, 2001; Griffiths *et al.*, 2013), center of expansion of C4 grasslands (Edwards *et al.*, 2010; Osborne and Sack, 2012).

Apochloa species showed a rustic Kranz anatomy, with the presence of enlarged BS cells. Although they presented a typical C3 leaf anatomy and most of the organelles (chloroplasts) are restricted to M cells, the percentage of

tissue related to BS cells may facilitate the establishment of C4 pathway in C4 genera related to these *Apochloa* species. Thus, more ultrastructural and phylogenetic investigations are needed in order to understand if those characteristics are coordinated with modifications of enzymes compartmentalization and the emergence of intermediate and C4 species in the clade related.

CONCLUSIONS

The presence of rustic Kranz anatomy in the *Apochloa* species analyzed could indicate facilitation to the development of the C4 photosynthesis in this group or may represent a trace of an ancestral C4 condition. However, ultrastructural and enzymatic analyses are required to confirm the photosynthetic metabolism of this species.

ACKNOWLEDGMENTS

This work was conducted during a scholarship supported by the “Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq” and financially supported by the “Research Support Foundation of Minas Gerais- FAPEMIG”.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

- Bianconi ME, Hackel J, Vorontsova MS, Alberti A, Arthan W, Burke SV, *et al.* Continued adaptation of C4 photosynthesis after an initial burst of changes in Andropogoneae grasses. *Syst Biol.* 2020;69(3):445-461. Doi: <https://doi.org/10.1093/sysbio/syz066>
- Brown WV. Leaf Anatomy in Grass Systematics. *Bot Gaz.* 1958;119(3):170-178.
- Castro NM, Menezes NL. Espécies de *Paepalanthus* Kunth, Eriocaulaceae da Serra Do Cipó (Minas Gerais). *Acta Bot Brasilica.* 1995;9(2):213-229.
- Christin P-A, Freckleton RP, Osborne CP. Can phylogenetics identify C4 origins and reversals? *Trends Ecol Evol.* 2010;25(7):403-409. Doi: <https://doi.org/10.1016/j.tree.2010.04.007>
- Christin P-A, Osborne CP, Chatelet DS, Columbus JT, Besnard G, Hodkinson TR, *et al.* Anatomical enablers and the evolution of C4 photosynthesis in grasses. *Proc Natl Acad Sci.* 2013;110(4):1381-1386. Doi: <https://doi.org/10.1073/pnas.1216777110>
- Christin P-A, Wallace MJ, Clayton H, Edwards EJ, Furbank RT, Hattersley PW, *et al.* Multiple photosynthetic transitions, polyploidy, and lateral gene transfer in the grass subtribe Neurachninae. *J Exp Bot.* 2012;63(17):6297-6308. Doi: <https://doi.org/10.1093/jxb/ers282>

- Dengler NG, Dengler RE, Donnelly PM, Hattersley PW. Quantitative Leaf Anatomy of C3 and C4 Grasses (Poaceae): Bundle Sheath and Mesophyll Surface Area Relationships. *Ann Bot.* 1994;73(3):241-255. Doi: <https://doi.org/10.1006/anbo.1994.1029>
- Dengler NG, Dengler RE, Hattersley PW. Differing Ontogenetic Origins of PCR (“Kranz”) Sheaths in Leaf Blades of C4 Grasses (Poaceae). *Am J Bot.* 1985;72(2):284-302. Doi: <https://doi.org/10.1002/j.1537-2197.1985.tb08293.x>
- Edwards EJ, Osborne CP, Strömberg CAE, Smith SA, Consortium CG. The Origins of C4 Grasslands: Integrating Evolutionary and Ecosystem Science. *Science.* 2010;328:587-591. Doi: <https://doi.org/10.1126/science.1177216>
- Edwards EJ, Smith SA. Phylogenetic analyses reveal the shady history of C4 grasses. *Proc Natl Acad Sci.* 2010;107(6):2532-2537. Doi: <https://doi.org/10.1073/pnas.0909672107>
- Ermakova M, Danila FR, Furbank RT, von Caemmerer S. On the road to C4 rice: advances and perspectives. *Plant J.* 2020;101:940-950. Doi: <https://doi.org/10.1111/tbj.14562>
- Giulietti AM, de Menezes NL, Pirani JR, Meguro M, Wanderley M das GL. Flora da Serra do Cipó, Minas Gerais: Caracterização e Lista das Espécies. *Bol Botânica da Univ São Paulo* 1987;9:1-151. Doi: <https://doi.org/10.11606/issn.2316-9052.v9i0p1-151>
- Giussani LM, Cota-Sánchez H, Zuloaga FO, Kellogg EA. A molecular phylogeny of the grass subfamily Panicoideae (Poaceae) shows multiple origins of C4 photosynthesis. *Am J Bot.* 2001;88(11):1993-2012. Doi: <https://doi.org/10.2307/3558427>
- Gowik U, Westhoff P. The Path from C3 to C4 Photosynthesis. *Plant Physiol.* 2011;155(1):56-63. Doi: <https://doi.org/10.1104/pp.110.165308>
- GPWG II. New grass phylogeny resolves deep evolutionary relationships and discovers C4 origins. *New Phytol.* 2012;193:304-312. Doi: <https://doi.org/10.1111/j.1469-8137.2011.03972.x>
- Griffiths H, Weller G, Toy LFM, Dennis RJ. You’re so vein: Bundle sheath physiology, phylogeny and evolution in C3 and C4 plants. *Plant Cell Environ.* 2013;36(2):249-261. Doi: <https://doi.org/10.1111/j.1365-3040.2012.02585.x>
- Hattersley PW, Watson L. C4 grasses: an anatomical criterion for distinguishing between NADP-malic enzyme species and PCK or NAD-malic enzyme species. *Aust J Bot.* 1976;24:297-308.
- Khoshravesh R, Stata M, Busch FA, Saladié M, Castelli JM, Dakin N, et al. The evolutionary origin of C4 photosynthesis in the grass subtribe Neurachninae. *Plant Physiol.* 2020;182:566-583. Doi: <https://doi.org/10.1104/pp.19.00925>
- Khoshravesh R, Stinson CR, Stata M, Busch FA, Sage RF, Ludwig M, et al. C3-C4 intermediacy in grasses: Organelle enrichment and distribution, glycine decarboxylase expression, and the rise of C2 photosynthesis. *J Exp Bot.* 2016;67(10):3065-3078. Doi: <https://doi.org/10.1093/jxb/erw150>
- Leegood RC. Roles of the bundle sheath cells in leaves of C3 plants. *J Plant Physiol.* 2008;59(7):1663-1673. Doi: <https://doi.org/10.1093/jxb/erm335>
- Lundgren MR, Dunning LT, Olofsson JK, Moreno-Villena JJ, Bouvier JW, Sage TL, et al. C4 anatomy can evolve via a single developmental change. *Ecol Lett.* 2019;22(2):302-312. Doi: <https://doi.org/10.1111/ele.13191>
- Lundgren MR, Osborne CP, Christin PA. Deconstructing Kranz anatomy to understand C4 evolution. *J Exp Bot.* 2014;65(13):3357-3369. Doi: <https://doi.org/10.1093/jxb/eru186>
- Mckown AD, Dengler NG. Key innovations in the evolution of Kranz anatomy and C4 vein pattern in *Flaveria* (Asteraceae). *Am J Bot.* 2007;94(3):382-389. Doi: <https://doi.org/10.3732/ajb.94.3.382>
- Medina BMO, Fernandes GW. The potential of natural regeneration of rocky outcrop vegetation on rupestrian field soils in “Serra do Cipó”, Brazil. *Rev Bras Botânica* 2007;30(4):665-678. Doi: <https://doi.org/10.1590/S0100-84042007000400011>
- Miyake H. Starch Accumulation in the Bundle Sheaths of C3 Plants: A Possible Pre-Condition for C4 Photosynthesis. *Plant Cell Physiol.* 2016;57(5):890-896. Doi: <https://doi.org/10.1093/pcp/pcw046>
- Morrone O, Agesen L, Scataglini MA, Salariato DL, Denham SS, Chemisquy MA, et al. Phylogeny of the *Paniceae* (Poaceae: *Panicoideae*): Integrating plastid DNA sequences and morphology into a new classification. *Cladistics* 2012;28(4):333-356. Doi: <https://doi.org/10.1111/j.1096-0031.2011.00384.x>
- Muhaidat R, Sage TL, Frohlich MW, Dengler NG, Sage RF. Characterization of C3-C4 intermediate species in the genus *Heliotropium* L. (Boraginaceae): Anatomy, ultrastructure and enzyme activity. *Plant Cell Environ.* 2011;34(10):1723-1736. Doi: <https://doi.org/10.1111/j.1365-3040.2011.02367.x>
- O’Brien TP, Kuo J. Development of the suberized lamella in the mestome sheath of wheat leaves. *Aust J Bot.* 1975;23:783-794. Doi: <https://doi.org/10.1071/BT9750783>
- Osborne CP, Sack L. Evolution of C4 plants: A new hypothesis for an interaction of CO₂ and water relations mediated by plant hydraulics. *Philos Trans R Soc B Biol Sci.* 2012;367(1588):583-600. Doi: <https://doi.org/10.1098/rstb.2011.0261>
- Rawsthorne S. C3-C4 intermediate photosynthesis: linking physiology to gene expression. *Plant Sci.* 1992;2:267-274. Doi: <https://doi.org/10.1111/j.1365-313X.1992.00267.x>
- Ribeiro KT, do Nascimento JS, Madeira JA, Ribeiro LC. Aferição dos limites da Mata Atlântica na Serra do Cipó, MG, Brasil, visando maior compreensão e proteção de um mosaico vegetacional. *Nat Conserv.* 2009;7(1):30-49.
- Sage RF, Christin PA, Edwards EJ. The C4 plant lineages of planet Earth. *J Exp Bot.* 2011;62(9):3155-3169. Doi: <https://doi.org/10.1093/jxb/err048>

- Sage RF, Khoshravesh R, Sage TL. From proto-Kranz to C4 Kranz: Building the bridge to C4 photosynthesis. *J Exp Bot.* 2014;65(13):3341-3356. Doi: <https://doi.org/10.1093/jxb/eru180>
- Sage RF, Sage TL, Kocacinar F. Photorespiration and the Evolution of C4 Photosynthesis. *Annu Rev Plant Biol.* 2012;63(1):19-47. Doi: <https://doi.org/10.1146/annurev-arplant-042811-105511>
- Sage RF. Tracking the evolutionary rise of C4 metabolism. *J Exp Bot.* 2016;67(10):2919-2922. Doi: <https://doi.org/10.1093/jxb/erw137>
- Sage RF. Environmental and evolutionary preconditions for the origin and diversification of the C4 photosynthetic syndrome. *Plant Biol.* 2001;3(3):202-213. Doi: <https://doi.org/10.1055/s-2001-15206>
- Voznesenskaya E V., Koteyeva NK, Akhani H, Roalson EH, Edwards GE. Structural and physiological analyses in *Salsola* (Chenopodiaceae) indicate multiple transitions among C3, intermediate, and C4 photosynthesis. *J Exp Bot.* 2013;64(12):3583-3604. Doi: <https://doi.org/10.1093/jxb/ert191>
- Zuloaga FO, Scataglioni MA, Morrone O. A phylogenetic evaluation of *Panicum* sects. *Agrostoidea*, *Megista*, *Prionitia* and *Tenera* (Panicoideae, Poaceae): Two new genera, *Stephostachys* and *Sorengia*. *Taxon.* 2010;59(5):1535-1546. Doi: <https://doi.org/10.1002/tax.595017>