JOYCE PEREIRA ALVARENGA

EVOLUTIONARY DIVERSIFICATION OF C_2 PHOTOSYNTHESIS IN THE GRASS GENUS *Homolepis* (ARTHROPOGONINAE)

LAVRAS – MG
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Prof. Dr. João Paulo Rodrigues Alves Delfino Barbosa
Orientador

Prof. Dr. Rowan F. Sage
Profa. Dra. Tammy L. Sage
Coorientador

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DIVERSIFICAÇÃO EVOLUCIONÁRIA DA FOTOSSÍNTESE C_2 NO GÊNERO DE GRAMÍNEAS *Homolepis* (ARTROPOGONINAE)

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós- Graduação em Agronomia/ Fisiologia Vegetal, área de concentração em Bioquímica, Nutrição e Metabolismo de plantas para a obtenção do título de Doutor.

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Dra. Rosy Mary dos Santos Isaias - UFMG

Dr. Rowan F. Sage - UofT

Dra. Tammy L. Sage - UofT

Dr. Vitor de Laia Nascimento - UFLA

Prof. Dr. João Paulo Rodrigues Alves Delfino Barbosa
Orientador

Prof. Dr. Rowan F. Sage
Profa. Dra. Tammy L. Sage
Coorientador

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RESUMO

O processo de evolução C_4, reconhecido como convergente, surgiu de forma suave e independente, de forma que novos traços foram reunidos muitas vezes em uma variedade de espécies. Além da diminuição das concentrações de CO_2, outros fatores ambientais, como aumento da temperatura, intensidade luminosa e escassez de água, contribuíram para induzir as modificações observadas nas espécies C_4. As características que surgiram e representaram uma vantagem foram então selecionadas. Importantes modificações anatômicas e bioquímicas ocorreram ao longo deste processo, e existem espécies que mantiveram tanto as características C_3 quanto C_4. Essas espécies intermediárias representam a ponte entre C_3 e C_4, e são de grande importância para estudos de evolução C_4. Há um grande número de gramíneas C_4 que representam culturas importantes. Estas são mais produtivas que espécies C_3; especialmente em ambientes com condições severas causadas por mudanças climáticas. Vale ressaltar, que a maioria das espécies C_4 são encontradas na família das gramíneas, porém estudos sobre a evolução C_4 e particularmente com espécies intermediárias estão concentrados nas eudicotiledôneas. Por esta razão, o objetivo deste trabalho foi identificar as características de gramíneas do gênero *Homolepis* quanto aos seus tipos fotossintéticos; e caracterizar suas células da bainha do feixe. Para isso, foram realizadas caracterizações anatômicas e de trocas gasosas em quatro espécies de *Homolepis*, *H. glutinosa*, *H. isocalycia*, *H. longispicula*, *H. aturensis* e uma espécie C_4 *M. loliiforme*, que pertence a um grupo próximo. Tanto os resultados anatômicos quanto fisiológicos demonstram claramente um gradiente nos tipos fotossintéticos. Além da espécie C_4 *M loliiforme*, foi encontrado uma C_3 *H. glutinosa,* uma Proto-Kranz *H. isocalycia* e duas C_2 *H. aturensis* e *H. longispicula*. Para a maioria das características anatômicas observa-se uma correlação positiva e forte entre elas e o ponto de compensação de CO_2. À medida que o ponto de compensação aumenta, com exceção da densidade de nervuras, a distância internerval, o número de células do mesofilo entre nervuras, a razão mesofilo: área do tecido da bainha do feixe e as dimensões das células da bainha do feixe também aumentam para espécies C_3. Nas espécies C_4 ocorre o inverso, enquanto as espécies intermediárias apresentam valores intermediários. Um resultado importante foram os valores encontrados para as células da bainha do feixe. Eles seguem um gradiente de C_3 a C_4, e a espécie C_3 tem as maiores dimensões celulares que a espécie C_4. No entanto, esta tendência é oposta ao que é discutido na literatura, necessitando de mais estudos deste tipo. Em conclusão, o presente estudo agrega informações importantes sobre o grupo *Homolepis*, contribuindo com a literatura sobre espécies intermediárias e nos processos evolutivos de assimilação de CO_2 em gramíneas.

Palavras-chave: Anatomia foliar. Evolução C_4. Fotossíntese. Poaceae. PACMAD.

ABSTRACT

The process of C_4 evolution, recognized as a convergent process, emerged smoothly and independently, in a way that novel traits were assembled many times in a variety of species. Besides the decrease in CO_2 concentrations, other environmental factors such as increases in temperature, light intensity and water scarcity, contributed to induce the modifications observed in C_4 species. Traits that emerged and represented a fitness advantage were then selected. Important anatomical and biochemical modifications occurred along this process, and there are species that maintained both C_3 and C_4 characteristics. These intermediate species represent the bridge between C_3 and C_4, and they have a great importance for studies on C_4 evolvement. There are a great number of important C_4 crops from the grass family. They come to be more productive than C_3 species; especially in environments with severe conditions caused by climate change. Noteworthy, the majority of C_4 species is found in the grass family, however studies on C_4 evolution and particularly with intermediate species are concentrated in eudicots. For this reason, the aim of this work was to identify the characteristics of species from the grass genus *Homolepis* regarding their photosynthetic types and characterize their bundle sheath cells. For that, anatomical and gas exchange characterization were conducted in four *Homolepis* species, *Homolepis glutinosa*, *Homolepis isocalycia*, *Homolepis longispicula*, *Homolepis aturensis* and one C_4 species *Mesosetum loliiforme,* that belongs to a sister group. Both anatomical and physiological results demonstrate clearly a gradient in the photosynthetic types. Besides the C_4 *M loliiforme*, it was found one C_3 *H. glutinosa*, one Proto-Kranz *H. isocalycia* and two C_2 *H. aturensis* and *H. longispicula*. For most of the anatomical traits it is observed a positive and strong correlation between them and the CO_2 compensation point. As the CO_2 compensation point increases, with the exception of veins density, the interveinal distance, the number of M cells between veins, the ratio mesophyll: bundle sheath tissue area and the bundle sheath cell measurements also increase for C_3 species. In C_4 species the opposite happens with lower values, while intermediate species have midway values. An important result was the values found for bundle sheath cells dimension. The results follow a gradient from C_3 to C_4, and C_3 species has bigger cells dimension than C_4 species. However, this trend is the opposite of what is discussed in the literature, hence requiring more studies of this kind. In conclusion, this current study aggregates important information about the *Homolepis* group, contributing with the literature about intermediate species and in the evolutionary processes of CO_2 assimilation in grasses.

Key-words: Leaf anatomy. C_4 evolution. Photosynthesis. Poaceae. PACMAD.

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1 INTRODUCTION

Approximately 30 million years ago, evolution of photosynthetic mechanisms culminated in the emergence of photosynthetic assimilation of carbon dioxide (CO_2) through C_4 pathway. The photosynthetic pathway evolved over 62 times in at least 18 families. This complex photosynthetic process results from a reorganization of anatomical and physiological characteristics of leaves, from species that assimilate CO_2 through the C_3 pathway (Christin, Osborne, et al., 2011; Sage et al., 2011). In general, the appearance of this C_4 pathway in several plants species is hypothesized to have been influenced by the drop in atmospheric CO_2 concentration. Values that were higher than 1500ppm in Cretaceous decreased to current values (below 400ppm) in Paleocene, after that a new increase occurred during Eocene and early Oligocene, but abruptly in late Oligocene decreased again reaching current concentrations (Christin et al., 2008; Ehleringer et al., 1991). However, in addition to the low concentration of CO_2, other crucial environmental factors such as increased temperature, water scarcity and higher radiation availability, may have also contributed to induce the physiological and anatomical modifications observed in C_4 species (Grass Phylogeny Working Group II., 2012; Sage et al., 2011, 2012).

The main characteristic that differentiates an ancestral C_3 from a C_4 species is the presence of a CO_2 concentrating mechanism around the active site of the enzyme Rubisco (Ribulose-1,5-bisphosphate Carboxylase Oxygenase), which has a dual affinity for CO_2 and oxygen (O_2). The environmental conditions that favor C_4 evolution are those that decreased CO_2 levels around the Rubisco catalytic site resulting in, an increase in Rubisco's activity as an oxygenase, the oxygenase activity of Rubisco results in a decline in photosynthesis. Hence, any mechanisms that enhance CO_2 concentration around the Rubisco catalytic site in C_4 photosynthesis, favors higher photosynthetic rates instead of photorespiration, increasing the carboxylation efficiency of Rubisco. In the majority of C_4 species there are a spatial division in which carbon fixation occurs at two moments. The first fixation occurs in the mesophyll (M) cells, by PEPc (Phosphoenolpyruvate Carboxylase) which has no affinity for O_2, while the second fixation occurs in the bundle sheath (BS) cells, by Rubisco. In the BS cells a molecule with four carbons, a product of the first CO_2 assimilation at M cells, will be decarboxylated and a molecule of CO_2 liberated. Thus, a higher concentration of CO_2 will be available around Rubisco improving its carboxylation activity (Bräutigam & Gowik, 2016; Sage et al., 2012).

C_4 photosynthesis has occurred many times and in many of those instances, it has been demonstrated that there is a gradual evolution in the development of the complex traits associated with C_4 photosynthesis. These traits include high vein density, enlarged cross-sectional area of BS cells, restriction of Rubisco to BS cells, loss of Glycine Decarboxylase Complex (GDC) from M cells and a reduction in M cells numbers between BS cells that reduce the distance for transport of metabolites between the two-celled photosynthetic process. Evidence of gradual evolution of the C_4 pathway is found in species that are known as intermediate species (C_3-C_4) because they have anatomical and physiological traits similar to C_3 and C_4 species (Schlüter & Weber, 2016). In some intermediate species, the mechanism of CO_2 concentration occurs through restriction of the enzyme GDC to BS cells. In this case, glycine originating from photorespiration in M cells will be decarboxylated at BS cells and a CO_2 molecule released, increasing the CO_2 to O_2 ratio in the Rubisco activation site. This process is known as C_2 photosynthesis, as the glycine molecule generated from this process is a 2-C molecule (Bräutigam & Gowik, 2016; Schlüter & Weber, 2016). However, other characteristics are also observed in C_3-C_4 species that promote a greater photosynthetic activity. Some of these features are an increase in vein density, decrease in volume (number and size) of M cells in relation to BS cells, and an increase in number and size of mitochondria and chloroplasts in BS cells (Sage et al., 2014; T. L. Sage et al., 2013; Yerramsetty et al., 2017). Considering the diverse combination of these multiple traits in different species, some authors have grouped intermediate species in different categories: C_3 proto-Kranz, C_2 (Type I and II) and C_4-like (Sage et al., 2014). The emergence of these CO_2 concentrating mechanisms established species better adapted to environments with higher temperature and lower water availability. These anatomical and physiological features observed in intermediate species, are suitable to maintain the photosynthetic capacity and integrity in such environments (Bräutigam et al., 2018).

The photosynthetic capacity of the plants has a great importance in plants growth, survival and reproduction. When considering crop plants, productivity will be hence directly correlated with its photosynthetic activity. Therefore, improvement of characteristics that increase plants CO_2 assimilation represents an important issue. With the Green Revolution, for example, there was a great increase in technologies and innovations, which culminated in improvements in plant productivity. After all the improvements already conquered, there has been stagnation in increasing plants productivity. Directly related to the processes of CO_2 assimilation, little has been done due

to its high complexity. Given that, the strategy of modifying the photosynthetic metabolism of C_3 plants emerges, so plants can be more efficient in CO_2 assimilation and achieve higher productivity, such as C_4 species, especially under adverse conditions, which could also improve the efficiency of inputs use (Schuler et al., 2016). Especially with climate change predictions and an increase in food demand in the next decades, it is necessary to invest in solutions that can contribute to increase crop productivity, rather than increasing farming areas. Therefore, a solution would be to improve and increase the efficiency of photosynthetic assimilation in crop plants that are not as efficient as C_4 crops.

For such accomplishment it is necessary to understand the evolution of the C_4 pathway characteristics. For studies of this kind, it is very important and wise to discover more intermediate species and study their anatomical traits and physiology, once they represent features between C_3 ancestral and fully functional C_4 species. Harada and coworkers studying an intermediate species (*Eleocharis vivipara*), that has C_4 traits in terrestrial environment and C_3 traits in submerged environment, provided important information, with *de novo* assembled sequence data, about observed genes that are related to C_4 leaf anatomy establishment. They founded in *E. vivipara* genes that are related to BS cells development and to changes in vascular and Kranz anatomy (Harada et al., 2018). Lyu and coworkers using molecular analysis to compare the expression of genes and proteins sequences between C_3, C_3-C_4 and C_4 species, found that there are some nodes reflecting critical moments where C_4 metabolism appears, including GDC reallocation and a C_4 cycle formation (Lyu et al., 2018). Other studies provide substantial information about genes that confer C_4 traits along the photosynthetic pathway evolution (Gowik et al., 2011; Kelly et al., 2017; Lundgren et al., 2019), understanding that this pathway through intermediates might be an important way to understand and discover the evolvement of such C_4 traits.

However, the great majority of the literature are largely dominated by eudicot species, due to the high number of intermediates within individual families that has been already found (Sage et al., 2014; Schulze et al., 2013). The relatively low number of intermediates in the monocots limits its investigations into evolution of the C_4 pathway, and constrains creation of theoretical models of C_4 evolution. Notably, it has been predicted that C_4 evolved differently in monocot than in eudicot, mainly by delaying or accelerating the acquisition of some C_4 anatomical and physiological features relative to eudicot species (Williams et al., 2013). Moreover, with the C_4

rice project and with major C_3 crops such as rice and wheat, studies with species that belongs to the same family, Poaceae, are essential and necessary.

For this reason, it is aimed to study the anatomical and physiological characteristics of grasses in the group PACMAD (Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae, Danthonioideae). It was chosen the group *Homolepis* that contains 5 species, and it is known to be a C_3 genus with a sister clade, *Mesosetum,* that contains C_4 species (Grass Phylogeny Working Group II., 2012). One species of this group, *H. aturensis* has been studied and discovered to be a C_2 species (Khoshravesh et al., 2016). Therefore, studying the other species could bring more information about the state of this group. With the results obtained, it will be possible to add more knowledge in the literature about intermediate species and in the evolutionary processes of CO_2 assimilation in grasses, contributing as well with projects such as the rice C_4 consortium.

2 LITERATURE REVIEW

2.1 CO_2 Concentrating Mechanism in the C_4 pathway

The main characteristic that differentiates an ancestral C_3 species from a C_4 species is the presence of a CO_2 concentrating mechanism, around the active site of the enzyme Rubisco that increases its carboxylation activity. In the majority of C_4 species there is a spatial division in which carbon fixation occurs at two phases. The first phase, occurs once CO_2 enters in the mesophyll cells (M) and is initially fixed into organic acids with four carbons (malate or aspartate). This reaction is driven by the enzyme Phosphoenolpyruvate Carboxylase (PEPc) that has no affinity for oxygen. The second phase occurs in the bundle sheath (BS) cells, where the product of four carbons from M cells is then decarboxylated, and a molecule of CO_2 is released and fixed at this time by Rubisco. This process allows an establishment of a higher CO_2 concentration compared to O_2 at the Rubisco reaction site. Hence, this mechanism promotes a decrease in the oxygenase activity of this enzyme, and therefore an increase in efficiency of CO_2 assimilation (Bräutigam & Gowik, 2016; Ehleringer et al., 1991; R. F. Sage et al., 2012). For the organization of this process, important characteristics are considered in this mechanism such as: the organization of M and BS cells, the transport of the organic acids from one compartment to another from M to BS cells, specific enzymes for

decarboxylation reaction - NADP malic enzyme (NADP-ME), NAD malic enzyme (NAD-ME) and Phosphoenolpyruvate carboxykinase (PPCK), and organization of cells surrounding vascular tissues forming the Kranz structure (Sage et al., 2014). The later feature is exclusively important for the maintenance of this concentrating mechanism. In some grasses, the cell layers forming the Kranz anatomy contains a differentiated cell wall similar to that of endodermis in roots with Casparian strips and suberin lamella, which creates a barrier for CO_2 diffusion from BS to M cells and intercellular spaces. The C_4 photosynthesis consists of a process of CO_2 assimilation by plants, in which the first stable molecule generated after CO_2 fixation, malate or aspartate, has 4-carbons. In C_3 photosynthesis, the reduction of CO_2 generates a molecule with 3-carbons, 3-PGA (3-Phosphoglycerate) (Rodrigues & Estelita, 2003; R. F. Sage et al., 2014).

2.2 Different Types of CO_2 Concentrating Mechanism

The process of C4 photosynthesis evolution occurred gradually, in a way that novel traits were not assembled directly, and ancestral genotypes were not all deleted. Traits that represent a fitness advantage will be selected (Sage et al., 2018). So, for a better comprehension about the evolution in C_4 pathway, for example, it is important to understand the characteristics that were added and maintained during the selection of the species along the way.

Some species are considered as intermediate (C_3-C_4) of the C_4 pathway evolution, because they have characteristics similar to C_4 species, such as the CO_2 concentrating mechanism, but still have characteristics of ancestral C_3 species, such as anatomical architecture and enzymes distribution. In general, as C_4 pathway appeared many times during the process of its evolution, there is a gradient in the development of characteristics of fully functional C_4 species, that is observed among the species called intermediates (Schlüter & Weber, 2016). In some intermediate species the mechanism of CO_2 concentration occurred through restriction of the enzyme Glycine Decarboxylase Complex (GDC) in BS cells, and the shuttle of glycine from photorespiration at M to BS cells. Through this restriction, glycine molecules are decarboxylated by GDC in BS cells, and a CO_2 molecule will be released at this site. In this case, there are also higher concentrations of Rubisco in BS cells, which will increase the assimilation of the released CO_2. This process is also known as C2 photosynthesis, because of the number of carbons in the glycine (2C) molecule

(Bräutigam & Gowik, 2016; Schlüter & Weber, 2016). Other characteristics observed in C_3-C_4 species are an increase in vein density, a decrease in volume (number and size) of M cells in relation to BS cells, an increase in number and size of mitochondria and chloroplasts in BS cells, and positioning of these organelles within the cells to facilitate CO_2 assimilation, (Khoshravesh et al., 2016; Sage et al., 2013; Yerramsetty et al., 2017). Given such diversity, some authors, considering the gradual change along the evolution of the C_4 pathway, have named other terms for species that may not fit the C_2 pathway, but have other characteristics than those already mentioned. These terms are Proto-Kranz, C_2 and C_4-type (C_4-like) (Khoshravesh et al., 2016; Sage et al., 2014).Species that are classified as proto-Kranz have an organelle repositioning and enrichment in BS cells during early C_4 evolution. There was a physiological activation of the BS cells of C_3 species by enhancing numbers and size of mitochondria and positioning of these organelles within the BS to facilitate refixation of photorespired CO_2 following decarboxylation of glycine with GDC (Khoshravesh et al., 2016; T. L. Sage et al., 2013).

Transition to an intermediate C_2 photosynthetic stage from Proto-Kranz is proposed to have resulted from continued amplification of centripetal mitochondria size and numbers in BS cells, BS cell-specific localization of GDC, increased chloroplast numbers in the BS, and a repositioning of most chloroplasts to the inner BS. Increases in vein density as well as BS cross-sectional area also occurs in tandem with Proto-Kranz and C_2 phases of C_4 evolution. These two later structural events involved alterations in both M and BS patterns of cell division and expansion (Khoshravesh et al., 2016; Sage et al., 2014; Sage et al., 2013). Traits closely related to C_4 species are considered characteristics of C_4-like species; such as, an increase in water and nitrogen use efficiency, activation of C_4 pathway enzymes (PEPc and NADP-malic enzyme) and a C_4 cycle occurring at BS cells, with a decrease of C_3 cycle in M species (Sage et al., 2014).

A recent work demonstrates a novel feature of the CO_2 concentrating mechanism in C_4 species. This mechanism involves the conversion of aspartate, synthesized in M cells, to oxaloacetate at BS cells, instead to malate. The molecule of oxaloacetate feeds the tricarboxylic acid cycle (TCA) in BS cells, then a molecule of 2-oxoglutarate leaves the cycle being converted to glutamate and right after decarboxylated to GABA (y-aminobutyric acid) by glutamate decarboxylase, resulting in a CO_2 molecule release (Kelly et al., 2017).

2.3 Differences in Gas Exchange

During the process of gas exchange, species that possesses a CO_2 concentration mechanism will exhibit a reduced CO_2 compensation point, while in C_3 species it will have higher values. Intermediate species which have a weak CO_2 concentrating mechanism will present a decrease in this value as well (R. F. Sage et al., 2014). Intermediate species (proto-Kranz and C_2) belonging to the genus *Flaveria*, have lower values of CO_2 compensation point when compared to *Flaveria* C_3 species. With this difference in CO_2 compensation point, can be inferred that Rubisco activity and efficiency might be enhanced in plants with lower values (Sage et al., 2013). Therefore, this characteristic in a context of environmental changes represents an advantage, especially in the presence of abiotic conditions that induce a decrease of CO_2 availability at the Rubisco active site are present.

2.4 The PACMAD Clade

Although many intermediate characteristics are already known, features related to the emergence and evolution of the C4 pathway has still been a central issue for plant biology. One of the gaps in knowledge occurs mainly in relation to the plant group that is the center of research in the present study. Although most C_4 species are found in the family Poaceae, monocot plants, many studies are conducted with species eudicot species, especially with the genus *Flaveria*, because they contain a large number of C_3-C_4 species that are already recognized as a model for the C_4 pathway evolution (Fisher et al., 2015; Gowik et al., 2011; Schulze et al., 2013).

Poaceae family is divided in two clades, PACMAD (Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae, Danthonioideae) and BEP (Bambusoideae, Ehrhartoideae, Pooideae). While in the clade BEP there are exclusively C_3 species, within the clade PACMAD the C_4 pathway has evolved multiple times in different origins (Christin et al., 2008; Grass Phylogeny Working Group II., 2012). This happens, for example, within the subtribe Arthropogoninae, belonging to the tribe Paniceae and subfamily Panicoideae, where several genera contain a significant variation in morphological, anatomical and physiological characteristics (Grass Phylogeny Working Group II., 2012; Morrone et al., 2012). The genera *Homolepis* represents an example of this variation, so far there are C_3 species e one discovered C_2 (*H.*

aturensis) (Grass Phylogeny Working Group II., 2012; Khoshravesh et al., 2016). Other sister groups of *Homolepis* have C_4 species *Arthropogon* and *Mesosetum*, and C_3 species *Apochloa* and *Canastra* (Morrone et al., 2012). Therefore, studies with species belonging to monocot are still needed once this group has plenty of plants to be studied and plants with commercial interest; including rice and wheat cultivars, which has great impact on food security along the globe.

2.5 Improving Photosynthetic Capacity

With the Green Revolution there was a great increase in technologies and innovations, which culminated with an increase in plant productivity. After all the improvements already made during this period, there has been stagnation in increasing plants yield. However, one of the processes that directly influence productivity is CO_2 assimilation, and, in this respect, few changes have been made due to the high complexity of this process. Given this, the strategy of modifying the photosynthetic metabolism of C_3 plants emerges as a solution to increase carboxylation efficiency and achieve higher yields, such as C_4 species, especially under adverse conditions, which could also improve the efficiency of inputs use (Schuler et al., 2016). With climate change predictions and the need to increase food demand in the next decades, it is necessary to invest in solutions that can contribute to increase crop productivity, rather than increasing farming areas. Therefore, a solution would be to improve and increase the efficiency of photosynthetic assimilation of crop plants.

Several studies have been conducted to understand C_4 photosynthetic metabolism, and to understand how its characteristics could be used in C_3 species to increase their yield and fitness facing the changes in the environment (Li et al., 2017; Lundgren et al., 2014; R. F. Sage & Zhu, 2011; Schuler et al., 2016; Sedelnikova et al., 2018). However, for such accomplishment, to transform plants metabolism, it is necessary to understand first the evolution of the C_4 pathway and how its traits evolved.

The present proposal aims to compare monocotyledon species from genera *Homolepsis* (C_3-C_2) and *Mesosetum* (C_4), to map characters of interest – anatomical, ultrastructural and physiological features – to understand the establishment of C_4 leaf anatomy and C_4 photosynthetic pathway, in the gradient found for intermediate species. With the results obtained, it will be possible to gather more knowledge in the literature about intermediate species, and about the C_4

pathway evolution. In order to understand the theory behind it, and also to increase the contribution with future research in plants breeding programs, as well with projects such as the rice C4 consortium.

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SECOND SECTION: MANUSCRIPT

SEARCHING FOR EVIDENCE OF INTERMEDIATE C_3–C_4 SPECIES IN THE GENUS *Homolepis*

ABSTRACT

The process of C_4 evolution, recognized as a convergent process, emerged gradually and independently, in a way that novel traits were assembled many times in a variety of species. There are intermediate species which possess both C_3 and C_4 characteristics and these are posited to be the bridge in the evolution from C_3 to C_4. The decrease in CO_2 concentrations, and other environmental factors such as increases in temperature, light intensity and water scarcity, are hypothesized to have enabled C_4 photosynthesis evolution. Important anatomical and biochemical modifications occurred to create the CO_2 concentrating mechanism that differentiates a C_3 to C_4. With climate change scenarios, studies on the evolution of C_4 traits have a great importance, because C_4 plants are more productive than C_3, especially in environments with severe conditions. More than that, intermediate species have this connection between the two photosynthetic types, then they are relevant to be investigated. Noteworthy, the majority of C_4 species is found in the grass family, however studies on C_4 evolution and with intermediate species are concentrated in eudicots. For this reason, the aim of this work was to identify the photosynthetic types characteristics of the grass genus *Homolepis* and characterize their bundle sheath cells. For that, anatomical and gas exchange characterization were conducted in four *Homolepis* species and one C_4 *Mesosetum* species. Anatomical and physiological results demonstrate clearly a gradient in the photosynthetic types from C_3, intermediates to C_4. Besides the characterization of the C_4 *M loliiforme*, it was found one C_3 one Proto-Kranz and two C_2 species. It is observed a lower value for the CO_2 compensation point (Γ), interveinal distance, number of mesophyll cells between veins, ratio mesophyll: bundle sheath tissue area and bundle sheath cell measurements for *M. loliiforme*, the C_4 species. While higher values are from the C_3 *H. glutinosa* and intermediate values are from intermediate species. Additionally, for the Proto-Kranz it is sometimes similar to the C_3, and for C_2 it is similar to the C_4 traits. The results found for bundle sheath cells measurements follows this gradient from C_3 to C_4, but it is the opposite from what is described in the literature. In this work, the bigger cells are from C_3 and the smaller from C_4 species. In conclusion, this current study aggregates important information about the *Homolepis* group, contributing with the literature about intermediate species and in the evolutionary processes of CO_2 assimilation in grasses. Moreover,

continuous studies are necessary with this group to understand about the emergence of those traits, and also regarding to the bundle sheath cells dimension.

Key-words: Leaf anatomy, C_4 evolution, Native species, Photosynthesis, Poaceae, PACMAD.

INTRODUCTION

The evolution of the photosynthetic assimilation of CO_2 through C_4 pathway occurred more than 60 times in different lineages since its inception approximately 30 million years ago (Christin et al., 2011; Sage, 2016). The multiple traits resulted from a reorganization of anatomical and physiological characteristics of leaves, from species that assimilate CO_2 through C_3 pathway. The main characteristic that differentiates an ancestral C_3 from a C_4 species is the presence of a CO_2 concentrating mechanism (CCM) around the active site of the enzyme Rubisco (Ribulose-1,5-bisphosphate Carboxylase Oxygenase), which has a dual affinity for CO_2 and O_2. In the majority of C_4 species there is a spatial division in which the first fixation occurs in the mesophyll (M) cells, by the cytosolic Phosphoenolpyruvate Carboxylase (PEPC), while the second fixation occurs in the bundle sheath (BS) cells, by Rubisco. In the BS cells a four carbon compound resulting from the primary CO_2 assimilation in M cells will be decarboxylated, and consequently increase CO_2 concentration around Rubisco enhancing carboxylation activity (Bräutigam & Gowik, 2016; R. F. Sage et al., 2012).

The evolution of the C_4 photosynthetic pathway is hypothesized to have occurred gradually with identifiable intermediate steps. Species with characteristics intermediate between C_3 and C_4 photosynthetic pathways are often called C_3-C_4 intermediates. Those intermediates have some common distinctive traits such as, a reduced CO_2 compensation point, different BS cells anatomy and organelles localization and a reduced photosynthesis sensibility to O_2. Some models predict that during the succession of events that proceeds the full C_4 cycle, it might start with the photorespiratory glycine pump (Schlüter & Weber, 2016; Stata et al., 2019). In this case, there is a CCM with a photorespiratory CO_2 recycling, which occurs through restriction of the enzyme Glycine Decarboxylase Complex (GDC) in BS cells. The glycine originated from photorespiration (process in which Rubisco fixes O_2) in M cells will be decarboxylated at BS cells and increase thus the CO_2 to O_2 ration on the Rubisco site; process called as C_2 photosynthesis (Bräutigam & Gowik,

2016; Monson & Moore, 1989; Schlüter & Weber, 2016). Nevertheless, before the shift in cell-specific enzyme localization there might be changes in the anatomy of BS cells. Such alterations include the anatomical and physiological functionalization of the BS cells, incremental reorganization of organelles in the different compartments, and the modification of vein density (Sedelnikova et al., 2018). These alterations promote a greater photosynthetic activity of these species specially in the habitat where they are found (Khoshravesh et al., 2016, 2020; R. F. Sage et al., 2014; Yerramsetty et al., 2017).

The appearance of the C_4 pathway in several plants species is hypothesized to have occurred mainly due to the drop in atmospheric CO_2 concentration in the past (Christin et al., 2008; Ehleringer et al., 1991). In addition, other crucial environmental factors such as increased temperature, water scarcity and higher radiation availability, may have also contributed to higher rates of photorespiration; and consequently the physiological and anatmical modifications observed in intermediate and subsequently C_4 species (Grass Phylogeny Working Group II., 2012; R. F. Sage et al., 2012, 2018).

Even though the C_4 photosynthetic pathway has appeared in many groups of plants, the grass family, Poaceae, is the major group with C_4 lineages. However, the great majority of the literature examining the gradual evolution of C4 photosyntesis is largely dominated by eudicot species, due to the high number of individual families that has been already found and studied with intermediate species (R. F. Sage et al., 2014; Schulze et al., 2013). The relatively low number of intermediates in the monocots limits its investigations into C_4 pathway, and constrains creation of theoretical models of C_4 evolution for this group (Williams et al., 2013). The grass family has two clades, BEP and PACMAD, and the C_4 evolution occurred repeatedly and independently only in the PACMAD clade (Christin et al., 2013; R. F. Sage et al., 2012). For this work it was chosen to study native species from the PACMAD clade with specific attention on the genus *Homolepis* that belongs to the tribe Paspaleae and the subtribe Arthropogoninae. This genus has five described species, *H. aturensis* (Kunth) Chase, *H. glutinosa* (Sw.) Zuloaga & Soderstr, *H. isocalycia* (G.Mey.) Chase, *H. longispicula* (Döll) Chase and *H. villaricensis* (Mez) Zuloaga & Soderstr, and they were believed to be C_3 (Giussani et al., 2001). However, one species *H. aturensis* has been well studied and described as a C_2 species (Khoshravesh et al., 2016), but confirmation determining the photosynthetic type of the other species has not been forthcoming. There is one genus, *Mesosetum,* which contains C_4 species and is the sister group of *Homolepis* (R. F. Sage et al., 2011),

which has also not been fully characterized. In this work it was chosen to study one species from *Mesosetum* group and four *Homolepis* species. In the present study, we focused on characterizing anatomical and photosynthetic physiological of the unstudied species to determine if any intermediate species are present in the genus *Homolepis*. Anatomical features we examined included traits related to photosynthetic activation of BS cells such as the increase in number of BS organelles specifically chloroplast, mitochondria and peroxisome; and changes in the three-dimensional features of M and BS cells, the ratio of M to BS cells, M-cell number and vein distance between M and BS cells as well as vein density (Monson & Moore, 1989; Sage et al., 2014). With the revealed information we could help to understand how this C_4 pathway evolved from steps of changes in the grass family; and contribute with future projects aiming the modification of C_3 crop species.

MATERIALS AND METHODS

Plant material

Native grasses from Brazil, belonging to the PACMAD group in the subtribe Arthropogoninae, were collected in the state of Minas Gerais – Brazil. These species were *Homolepis glutinosa* (Sw.) Zuloaga & Soderstr, *Homolepis isocalycia* (G.Mey.) Chase, *Homolepis longispicula* (Döll) Chase and *Mesosetum loliiforme* (Hochst.) Chase (Field coordinates in Supplementary Material – TABLE S1). Another species *Homolepis aturensis (Kunth) Chase* was obtained from UofT collection (from Costa Rica).

Plants were grown at the University of Toronto (Canada) in a greenhouse with controlled day/night temperatures of 30°C/25°C. The species were planted in 10L pots of a sandy-loam soil. For the species *H. longispicula* and *M. loliiforme,* which were collected in the rocky field area (characterized by the occurrence of gravel and sand), it was used a different soil that comprises a mixture of sand, turface and topsoil. Plants were watered daily, and fertilized weekly with a 1:1 mixture of Miracle-Grow 24-10-10 All Purpose Plant Food and Miracle Grow Evergreen Food (30-10-20), with addition of iron EDDHA, magnesium sulphate and calcium nitrate solution.

Gas exchange analysis

Gas exchange measurements were conducted for 4 *Homolepis* species, *Mesosetum loliforme, and* 2 other PACMAD grasses as reference, *Phragmites australis* (Cav.) Trin. ex Steud. (C_3) and *Anthephora pubescens* Nees. (C_4). Gas exchange of intact fully expanded leaves was determined using a LiCor 6400 gas exchange system. Measurements were done along the day with leaf temperature at 30 ± 1 °C and a leaf vapor pressure deficit (VPD) of 2 ± 0.4 kPa. For measurement of the response of net CO_2 assimilation rate (A) to intercellular CO_2 concentration (Ci) at light saturation (A/Ci curves), leaves were first equilibrated at ambient CO_2 concentration of 400 μmol m^{-2} s^{-1} and at light intensity of 1250 for *H. isocalicya* and 1750 μmol photons m^{-1} s^{-1}, for other species. The levels of ambient CO_2 were slowly reduced in steps, until reach values of 25 for C_2 and C_4 species or 50 μmol m^{-2} s^{-1} for C_3 species, with measurements being done at each step after rate equilibration. After that, CO_2 value was then returned to 400 μmol m^{-2} s^{-1} and increased in steps to reach higher values of 1500 μmol m^{-1} s^{-1}, with measurements made at each step.

The linear initial slope of the A/Ci response was used to estimate the CO_2 compensation point (Γ). For leaf gas exchange, 4–15 measurements were conducted on 3–5 plants per species. The carboxylation efficiency (CE, μmol m^{-1} s^{-1} μmol m^{-2} s^{-1}) was obtained as the equivalent to the initial slope of the A/Ci response. The intrinsic water use efficiency (A/gs) was calculated dividing the stomata conductance (gs) by A.

Leaf anatomy, ultrastructure, and immunolocalization

Leaf anatomy was characterized on the most recent, fully expanded leaf. Leaf vein density and BS morphology was quantified on cleared leaves and internal leaf anatomy was assessed using light (LM), confocal (CM) and transmission electron microscopy (TEM). Leaves were cleared for vein measurements in a 3:1 of ethanol:acetic acid for 24h, placed in 0.5M NaOH (sodium hydroxide) for 24h, and subsequently placed in 50% bleach until transparent. Leaves used for determining BS morphology were further rinsed 3 times in water, stained overnight in 0.6% Calcofluor White and cleared in 90% TDE (2,2'-thiodiethanol; Hasegawa et al., 2016) before mounting slides. Cleared leaf tissue was mounted in antifade slides and z-stacks of cleared tissue

were imaged with a Leica TCS SP8 with a Diode 405 laser. A Zeiss Axiophot light microscope equipped a DP71 Olympus camera and Olympus CellSens imaging software (Advanced Microscopy Techniques, MA, USA) and a Phillips 201 transmission electron microscope equipped with an Advantage HR camera system (Advanced Microscopy Techniques, Wobum, MA, USA) were used to capture images of clearings and resin-embedded leaf sections.

Anatomical and Morphological Parameters Quantified and Statistical Analyses

For characterization of vein density, BS morphology and internal leaf anatomy, leaf samples were collected from 4 plants. For all traits measured, the values per plant were averaged to give one value per plant. These individual plant values were the unit of replication for statistical analysis. For characterization of vein density, for each biological replicate it was taken 4 pictures from different areas of the leaf. With these images the length of all veins present in each image were added together, and the average of these four measurements represented one replicate plant. Bundle sheath morphology was characterized by using z-stacks to quantify the volume, area, and length (parallel to vascular tissue) and width (perpendicular to vascular tissue) of xylem and phloem associated BS cells. Paradermal and cross sections obtained from resin-embedded leaf tissue were also used to quantify BS area, length, and width of xylem and phloem associated BS cells. Each parameter was measured on 20 cells per biological replicate. Cross sections were also used to determine the interveinal distance (IVD), number of MS cells between veins, MS tissue area, BS tissue area and ratio of BS/MS tissue area. For each parameter it was taken 10 measures in different images per biological replicate. All measurements were taken using ImageJ software.

The data from all anatomical measures follows normal distribution, therefore they were submitted to one-way ANOVA followed by a Scott Knott's post-hoc test ($p<0.05$).

Starch Extraction for Carbon Isotope Ratio

Leaf samples from all species were collected, frozen in liquid nitrogen and stored at -80°C for later starch extraction and δ13C measurements. The starch extraction and hydrolysis were done using methanol: chloroform: water (12:5:3, v/v/v) (MCW), and amylase solution in water

according to the method described by Richter et al. (2009). It was used 150 µL of the extract in tin capsules (model D1006, Elemental Microanalysis, Okehampton, UK) with 50µL increments and evaporated. It was also measured δ13C of dried leaf samples from all species. All δ13C measurements were conducted by the Washington State University Stable Isotope Core. The samples consisted of 3 biological replicates for each species the unit of replication for statistical analysis

Data analysis

Results were analyzed using the R software (packages: ExpDes.pt, ggplot2, Hmisc, Rmisc, rstatix, agricolae, dplyr, tidyverse, grid and gridExtra) (R Core Team 2020). For normalized data we submitted it to one-way ANOVA followed by a Scott Knott's post-hoc test ($p<0.05$), and for data that did not follow a normal distribution we used Kruskal-Wallis test, followed by the post-hoc Dunn's Test ($p<0.05$). The data obtained for gas exchange measures did not follow a normal distribution, therefore we used Kruskal-Wallis test, followed by the post-hoc Dunn's Test ($p<0.05$) to see the differences between species.

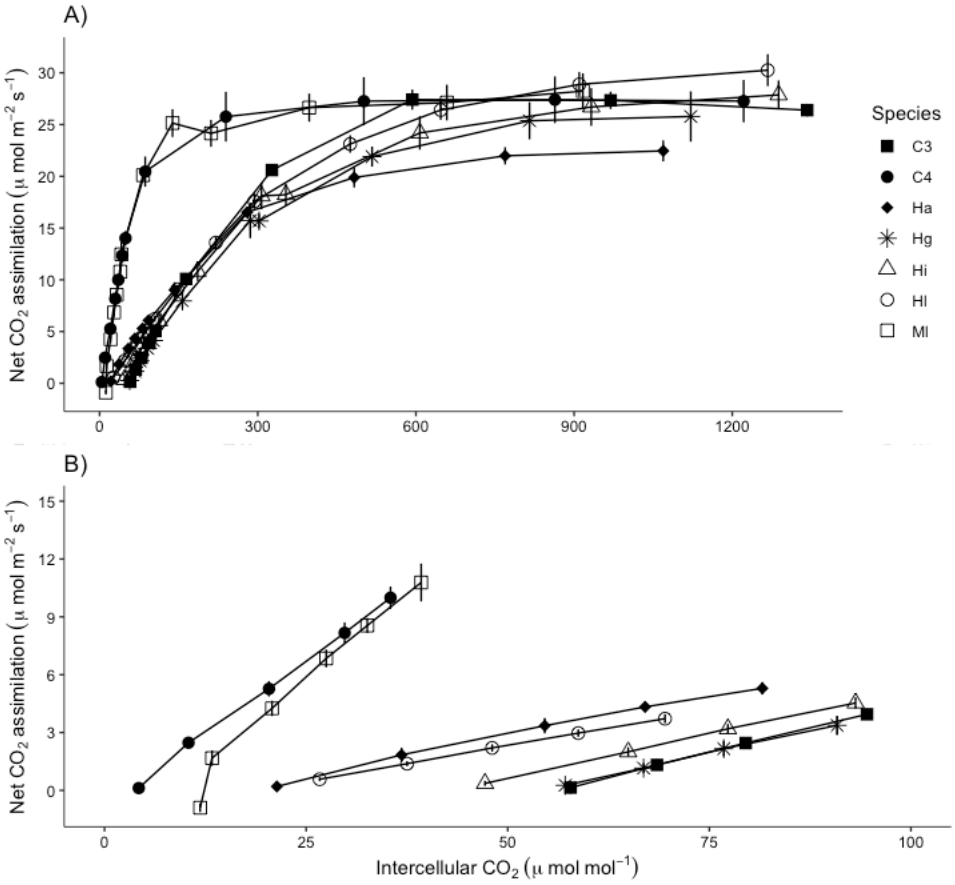
RESULTS

Photosynthetic gas exchange

The net CO_2 assimilation rate (A) in response to intercellular CO_2 partial pressure (Ci) (A/Ci curve) provides the CO_2 compensation point (Γ). This is an important parameter for discrimination between the different carbon concentration mechanisms. Typically, Γ values are 50-60 µmol CO_2 mol^{-1} air for C_3 species, 10-40 µmol CO_2 mol^{-1} air for intermediate species, and close to zero µmol mol^{-1} for C_4 species (Sage et al., 2014). The response of A/Ci curves for *H. glutinosa* and *P. australis* is typical of C_3 species, showing a Γ of 54.50 µmol CO_2 mol^{-1} air and 55.94 µmol CO_2 mol^{-1} air, respectively. Differently, the Γ for C_4 species was 8.34 µmol CO_2 mol^{-1} air for *M. loliiforme* and 3.30 µmol CO_2 mol^{-1} air for *A. pubescens*. Between C_3 and C_4 values there are the intermediate Γ values of 43.08 µmol CO_2 mol^{-1} air for *H. isocalycia*, 17.83 µmol CO_2 mol^{-1} air for *H. aturensis* and 17.84 µmol CO_2 mol^{-1} air for *H. longispicula* (FIGURE 1, TABLE 1 and

Supplementary FIGURE S1). In Figure 1B this difference among intermediate Γ values is more prominent, it is seen a spatial separation among the lines. The two intermediate species with lower Γ, *H. longispicula* and *H. aturensis*, are aligned while *H. isocalycia* is closer to C_3 species. For both C_4 species there is a steeper initial slope in assimilation at a lower Ci, this is a classical functioning in C_4 curves due to its mechanism for CO_2 concentration (FIGURE 1).

Figure 1- Response of net CO_2 assimilation rate to variation in intercellular CO_2 concentration for four *Homolepis* species, *M. loliiforme*, *Phragmites australis* (C_3 reference), and *Anthephora pubescens* (C_4 reference). A) Complete A/Ci curves. B) The initial slope of A/C_i curves at low Ci values. Mean with SE, n=3 for *M. loliiforme, P. australis* and *A. pubescens* and n=5 for other species. C3 - *P. australis,* C4 – *A. pubescens,* Ha – *H. aturensis,* Hg – *H. glutinosa,* Hi – *H. isocalycia,* Hl – *H. longispicula* and Ml – *M. loliiforme.*



Source: From author (2022).

Other variables were obtained through the A/Ci curves (TABLE 1 and Supplementary material FIGURE S1). The carboxylation efficiency of Rubisco (CE, μmol m^{-1} s^{-1} μmol m^{-2} s^{-1}) was acquired as the equivalent to the initial slope of the A/Ci response. The average value for *M. loliiforme* was 0.36 and for *A. pubescens* was 0.31; indicating higher values when compared to the other species that have values around 0.1. Considering these numbers, there is a decrease of 72% in CE in species that does not have the C_4 pathway; specifically, *H. longispicula* has the lowest CE value of 0.07 (TABLE 1). Regarding to A at 400 μmol CO_2 mol^{-1} air and the intrinsic water use efficiency (estimated as A/gs at 400 μmol CO_2 mol^{-1} air), C_4 species also have the highest values. While intermediate species results are found between C_3 and C_4. The results found for the stomata conductance (gs) are responsible as well, for these last variables. The C_4 *M. loliiforme* has the lowest gs, the highest value is from *P. australis* the reference C_3 and the reference C_4 *A. pubescens* has similar value to the intermediate *H. longispicula* and *H. glutinosa* (TABLE 1, Supplementary material FIGURE S1).

Table 1 - Summary of leaf gas exchange results for four *Homolepis* species, *M. loliiforme* and two other reference grasses, *Phragmites australis* C_3 and *Anthephora pubescens* C_4. Values represent median and interquartile range (Iqr), n= 3 for *M. loliiforme*, *P. australis* and *A. pubescens*, and n= 5 for other species. In each column, values followed by the same letter are not different (Kruskal-Wallis test, followed by the post-hoc Dunn's Test ($p<0.05$)). 400 refers to ambient CO_2 concentration in µmol mol^{-1}.

Species	A at 400 (µmol m^{-2} s^{-1})	Γ (µmol mol^{-1})	CE	A/gs (µmol mol^{-1})	gs (µmol m^{-2} s^{-1})
***Phragmites australis* - C_3**	20.80 (0.508) ^{ab}	55.90 (0.989) ^{a}	0.10 (0.01) $_{ab}$	25.20 (0.79) ^{c}	0.82 (0.06) ^{a}
Homolepis glutinosa	14.80 (2.71) ^{b}	55.10 (0.66) ^{a}	0.08 (0.05) $_{ab}$	41.10 (2.54) ^{abc}	0.36 (0.03) ^{ab}
Homolepis isocalycia	17.60 (0.93) ^{ab}	43.10 (0.89) ^{ab}	0.09 (0.01) $_{ab}$	39.50 (2.96) ^{bc}	0.45 (0.01) ^{a}
Homolepis longispicula	17.60 (1.20) ^{ab}	18.50 (5.53) ^{abc}	0.07 (0.02) $_{b}$	52.90 (4.27) ^{abc}	0.33 (0.01) ^{ab}
Homolepis aturensis	16.00 (1.36) ^{b}	15.60 (3.71) ^{abc}	0.09 (0.01) ^{ab}	32.30 (7.47) ^{bc}	0.49 (0.15) ^{a}
Mesosetum loliiforme	25.10 (0.96) ^{a}	9.41 (1.27) ^{bc}	0.39 (0.03) ^{a}	149.00 (2.68) ^{a}	0.17 (0.01) ^{b}
***Anthephora pubescens* - C_4**	25.80 (2.57) ^{a}	3.30 (0.62) ^{c}	0.31 (0.04) ^{a}	71.00 (6.51) ^{ab}	0.36 (0.08) ^{ab}

Source: From author (2022).

Carbon isotope ratio

The carbon isotope discrimination data have been used for a long time to differentiates between C_3 and C_4 species, and it is a reliable attribute. However, as it occurs with other traits this variable does not differentiates well intermediate species as Γ values does (Schlüter & Weber, 2016). For this work, it was analyzed both starch and bulk leaves carbon isotope ratios. While C_3 and intermediate species results varied from -32.71‰ to -29.15‰ from bulk leaves and -27.52‰ to -24.61‰ from starch material; *M. loliiforme* values were -13.37‰ (bulk leaves) and -16.75‰ (starch) (TABLE 2).

Table 2 - Carbon isotope ratio from bulk leaves and starch material of four *Homolepis* species and *M. loliiforme*. Mean ± SE, n=4. Letters indicate common statistical groups according to one-way ANOVA followed by a Scot-Knots's post-hoc test ($P \leq 0.05$).

Species name	$\delta^{13}C$ (‰) Bulk leaves	$\delta^{13}C$ (‰) Starch
H. glutinosa	-29,15 ± 0.13 d	-24.61 ± 0.39 b
H. isocalycia	-30.55 ± 0.02 b	-27.52 ± 0.14 a
H. longispicula	-29.57 ± 0.04 c	-25.04 ±0.60 b
H. aturensis	-32.71 ±0.01 a	-26.75 ±0.43 a
M. loliiforme	-13.37 ± 0.16 e	-16.75 ± 0.80 c

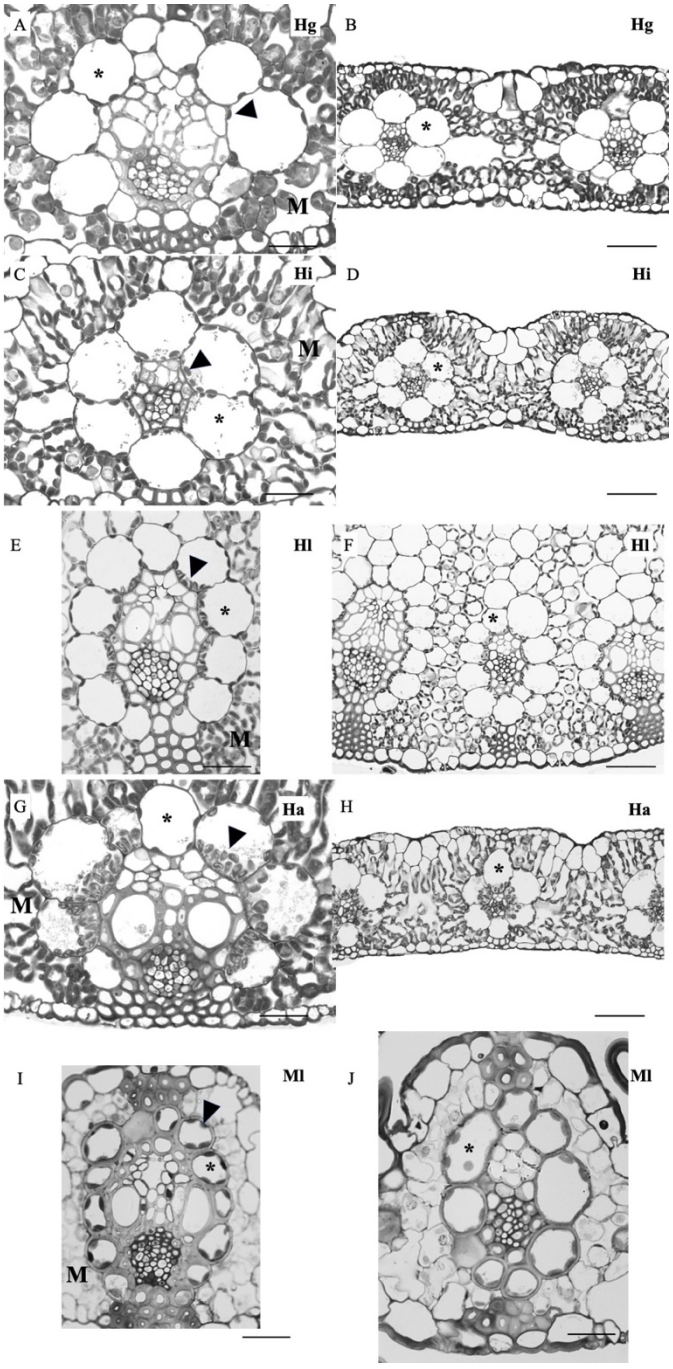
Source: From author (2022).

Leaf anatomy parameters

With respect to the external leaf morphology of *Homolepis* species, *H. glutinosa*, *H. isocalycia* and *H. aturensis* have the same shape. Regarding the size, *H. glutinosa* is the biggest, *H. isocalycia* and *H. aturensis* have the same size, it is even hard to distinguish them. On the other hand, *H. longispicula* has a long, thin and thicker leaf, very distinctive from the others (Supplementary material FIGURE S2).

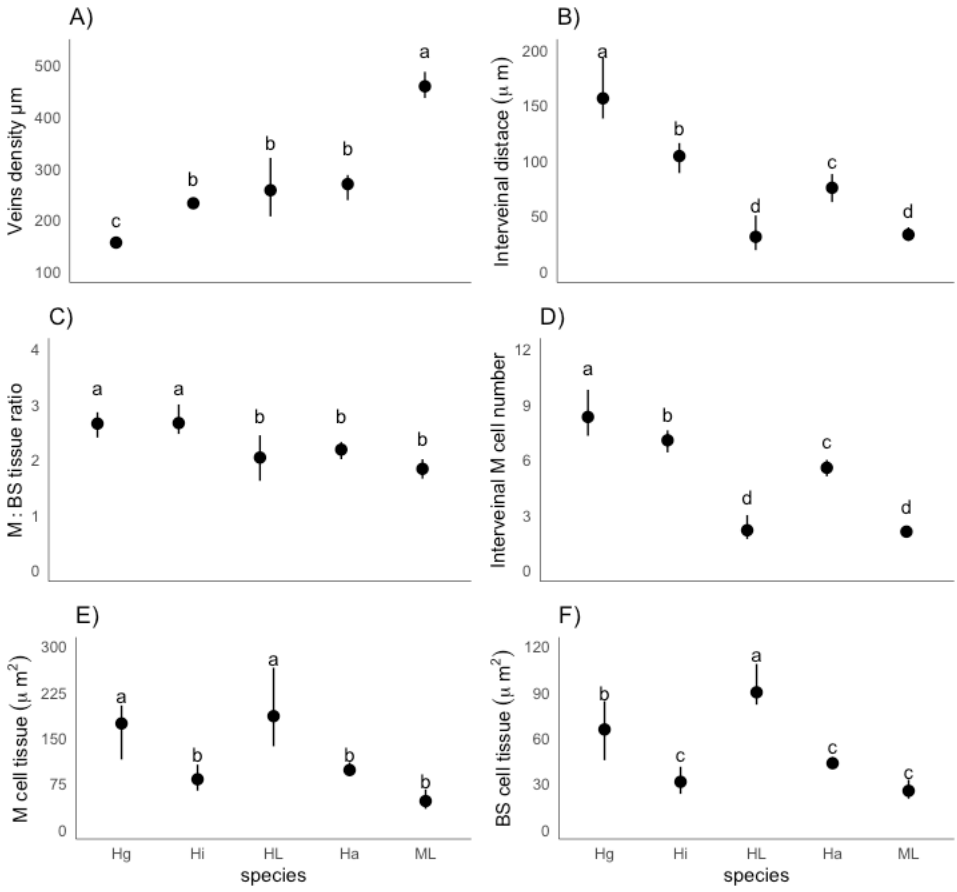
With cross sections images it was possible to examine the leaf anatomy of the species, and measure traits that are usually considered to associate photosynthetic pathways. With the exception of *H. longispicula*, all *Homolepis* species studied here have around 3 layers of M cells between the adaxial and abaxial epidermis, and a palisade mesophyll, whereas *H. longispicula* has more than 10 M cell layers and does not have palisade tissue (FIGURE 2). This higher number of M cells layers in *H. longispicula* contributed to an increase in leaf thickness. Observing the traits that are mostly studied to compared photosynthetic pathways; the values found for IVD and number of M cells between veins follow a gradient with *M. loliiforme* and *H. longispicula* representing the lowest values and with steps increasing there are *H. aturensis, H. isocalycia* and *H. glutinosa* respectively (FIGURE 3B and D). The results of M and BS tissue area, and its ratio M:BS are statistically the same for *M. loliiforme* and all intermediate species, except *H. longispicula*. For *H. glutinosa* these values were higher, typically of C_3 species; and once *H. longispicula* has thicker leaves their values are similar to C_3 pattern as well (FIGURE 3C, E and F). Regarding vein density, the C_4 *M. loliiforme* has the highest value, *H. glutinosa* the lowest and the remaining *Homolepis* species have an intermediate vein density and they are statistically similar (FIGURE 3A). Considering the classification of veins, C_3 and intermediate species have the midvein, lateral veins and rank 1 veins; and *M. loliiforme* has all these veins plus rank 2 veins (Supplementary material FIGURE S3). Counting the veins of the entire leaf it is observed that the high density of C_4 veins in this study is located between the midvein and lateral 3 vein. For *H. glutinosa* it is observed 4 rank 1veins between midvein and lateral 1 vein, while *M. loliiforme* has double this number (rank 1 and rank 2) (Supplementary material TABLE S2). Whereas, the portion that contains the margins of the leaf in *M. loliiforme* has the same amount of veins as the other species. Comparing just the total of lateral veins and rank 1 and 2 veins among the species is not correct, once the width of the leaves is different. Then, considering the ratio number of total lateral veins: leaf width and number of total rank 1 and 2 veins: leaf width, the C_4 and intermediates species have a higher ratio than C_3 species, indicating the gradient again in vein density where C_3 has less veins than C_4 species (Supplementary material TABLE S2 and FIGURE S4).

Figure 2 - Leaf transverse sections demonstrating the gradient found in traits from C_3 to C_4 species. Specially bundle sheath cells, organelles distribution and distance between veins from A, B) *H. glutinosa*; C, D) *H. isocalycia*; E, F) *H. longispicula*; G, H) *H. aturensis* and I, J) *M. loliiforme*. M, mesophyll; asterisk, bundle sheath and arrow heads organelles. Scale bar left side = 20 µm, right side = 50 µm.



Source: From author (2022).

Figure 3 - Leaf anatomical characteristics from cross section images of *Homolepis* and *Mesosetum* species. A) Veins density (10^{-2}); B) Interveinal distance; C) M:BS tissue area ratio; D) Number of mesophyll cells between veins; E) M tissue area (10^{-2}) and F) BS tissue area (10^{-2}). Points indicate the mean with min and max; n=4. Letters indicate common statistical groups according to one-way ANOVA followed by a Scot-Knots's post-hoc test ($P \leq 0.05$). Hg – *H. glutinosa,* Hi – *H. isocalycia,* HL – *H. longispicula,* Ha – *H. aturensis* and ML – *M. loliiforme.*



Source: From author (2022).

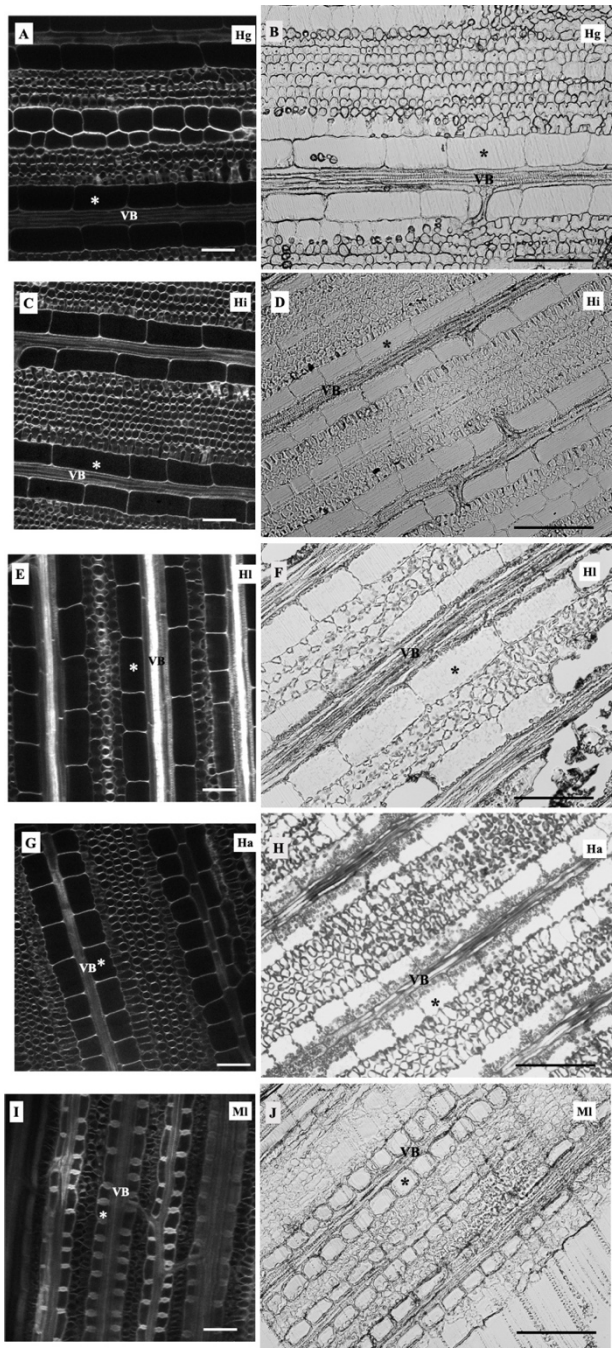
In this work, besides cross sections it was also conducted confocal imaging with longitudinal view for measurements of BS cells, which is an innovative technique for this field (FIGURE 4). It was found that *H. glutinosa* BS cells volume and area are 14 and 7 times bigger, respectively, than *M. loliiforme*. Statistically, *H. aturensis* BS cell area is 3 times bigger than *M. loliiforme*, however their BS cell volume is the same. The other intermediates, *H. isocalycia* and *H. longispicula*, have the same volume and area, and their volume is 2 times bigger than *H.*

aturensis and 2 times smaller than *H. glutinosa* (FIGURE 4 and 5). Regarding the BS cells length and width, all *Homolepis* species have greater values than *M. loliiforme.* Among them, in length *H. glutinosa* is the longest and *H. aturensis* the shortest. While in width, *H. glutinosa* and *H. longispicula* are the largest, and *H. isocalycia* and *H. aturensis* the smallest. The ratio of length and width (L:W) is not statistically different between species, but there is a strong (R=0.87) and positive correlation between length and width of the species (Supplementary material FIGURE S5). These dimensional variations result in more BS cells along the length of the vascular strands of intermediate and C_4 compared to C_3 plants (FIGURE 4).

Those significant differences in anatomical parameters between species were subjected to a correlation test with the compensation point of the species; and it was found a relationship between them (FIGURE 6). The results show a strong correlation with most of the traits observed in Figure 3 and 5. As Γ value increases, the IVD, the number of M cells between veins, the ratio M:BS tissue area and the BS cells measurements also increases for C_3 species. On the other hand, a higher Γ indicates a lower value for veins density. For isolated M and BS tissue area the correlation was not significative.

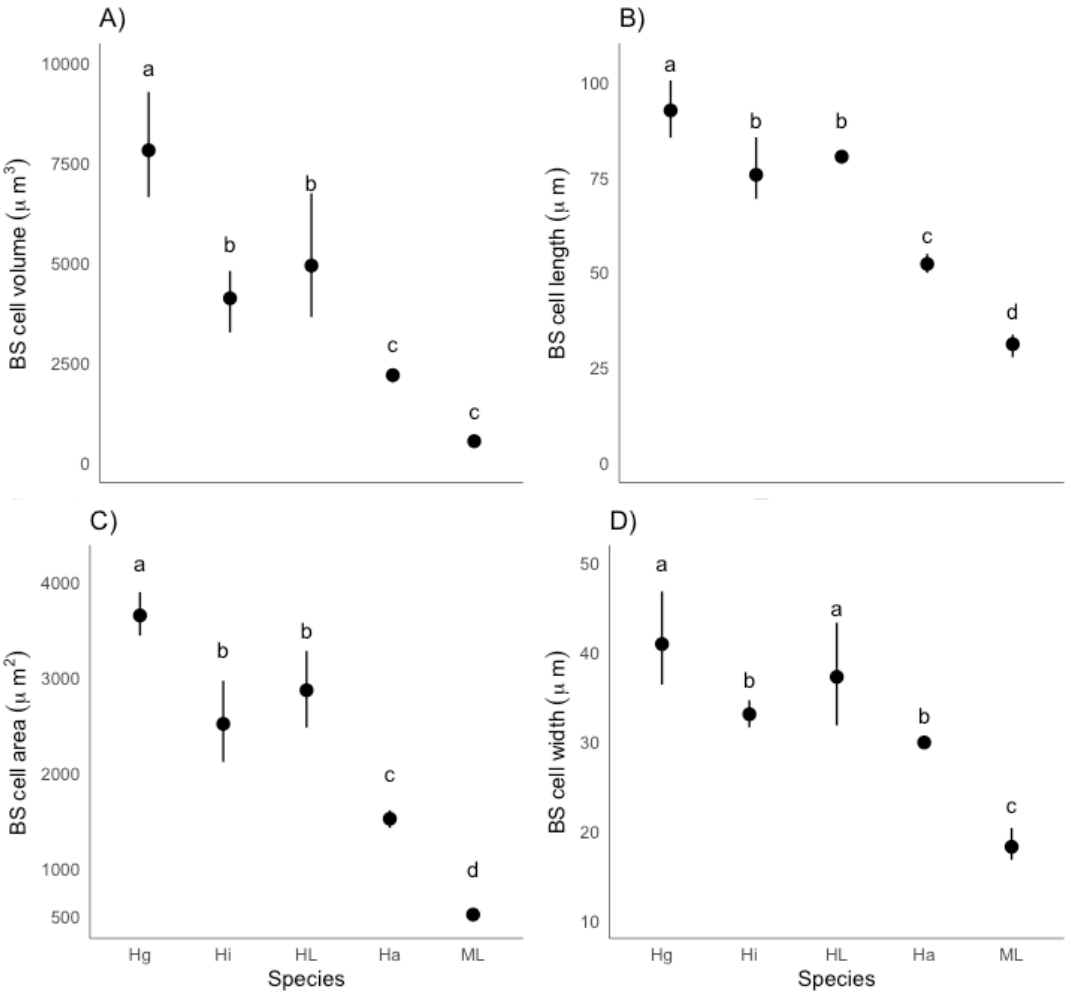
The organelle content observed in BS cells of *Homolepis* and *M. loliiforme* species forms a gradient in the amount and its location (FIGURE 2 and 7). In *H. glutinosa* there are few organelles dispersed around the cell wall of BS cell. While *M. loliiforme* organelles are numerous and located in the centrifuge position of BS cells, typical of C_4 species (FIGURE 2A, E, 7A and B). Among the intermediate species *H. isocalycia* has lesser organelles and while they are dispersed around the cell wall, qualitatively to be more mitochondria adjacent the vein; organelles in *H. longispicula* and *H. aturensis* are numerous and have moved from an even distribution around the cell to an organized centripetally position adjacent to the vascular tissue (FIGURE 7C - F). It is observed that in intermediate species mitochondria are localize in the centripetal BS cells pole surrounded by chloroplasts. Some other grasses have their organelles concentrating in the inner sheath, MS (Lundgren et al., 2014), but for *Homolepis* this is not the case. Differently of what is seem in BS cells the organelles are distributed similar in the M cells of all species. Organelles are positioned in the cell periphery facing the intercellular air space and other M cells (FIGURE 7).

Figure 4 - Leaf longitudinal sections from confocal microscopy (left side) and light microscope (right side) demonstrating the differences in bundle sheath cell sizes. A, B) *H. glutinosa*; C, D) *H. isocalycia*; E, F) *H. longispicula*; G, H) *H. aturensis* and I, J) *M. loliiforme*. Asterisk, bundle sheath cell; VB, vascular bundle. Scale bar: left side = 50 μm and right side = 100 μm.



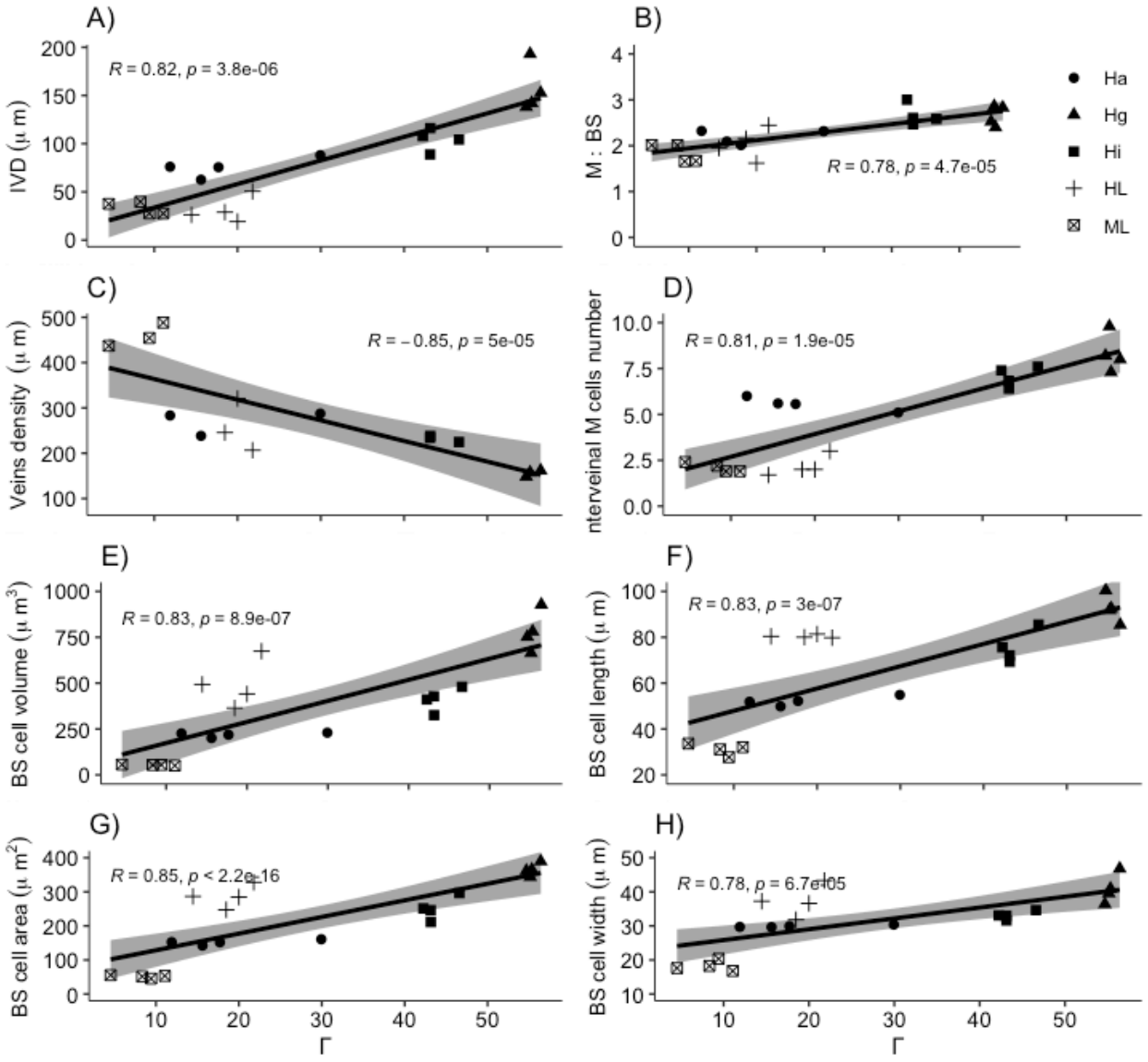
Source: From author (2022).

Figure 5 - Measurements of bundle sheath cells from confocal microscopy of segmented leaves of *Homolepis* and *Mesosetum* species. A) Bs cells volume (10^{-1}); B) BS cells length; C) BS cells area and D) BS cells width. Points indicate the mean with min and max; n=3. Letters indicate common statistical groups according to one-way ANOVA followed by a Scot-Knots's posthoc test ($P \leq 0.05$).). Hg – *H. glutinosa*, Hi – *H. isocalycia*, Ha – *H. aturensis*, HL – *H. longispicula* and ML – *M. loliiforme*.



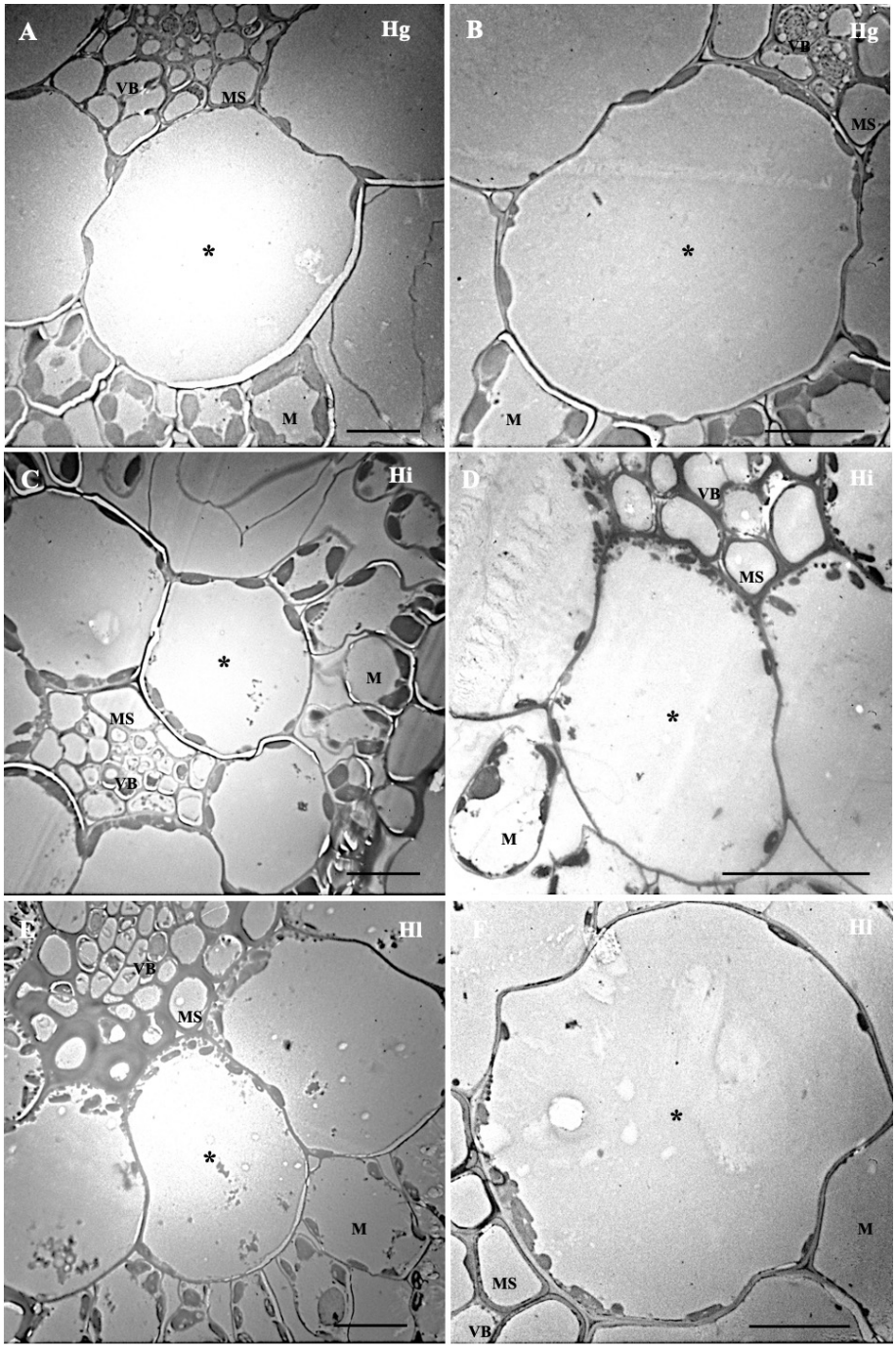
Source: From author (2022).

Figure 6 - Relationship between gamma and anatomical traits. A) Interveinal distance; B) M:BS tissue area; C) Veins density; D) Number of M cells between veins; E) BS cells volume (10^{-2}); F) BS cells length; G) BS cells area (10^{-1}) and H) BS cells width. Method of correlation Spearman. Significant correlations at $p \leq 0.05$ are shown. Ha- *H. aturensis*; Hg- *H. glutinosa*; Hi- *H. isocalycia*; HL- *H. longispicula* and ML- *M. loliiforme*.



Source: From author (2022).

Figure 7 - Transmission Electron Microscopy (TEM) images of BS cells with its gradient in organelle aggregation. A, B) *H. glutinosa;* C, D) *H. isocalycia* and E, F) *H. longispicula.* Scale bar = 10 µm. Asterisk, bundle sheath cell; VB, vascular bundle; M, mesophyll.



Source: From author (2022).

DISCUSSION

Characterizing *Homolepis* photosynthetic types

This study presents a comprehensive analysis of the physiological and anatomical parameters of the different photosynthetic types in the grass genus *Homolepis*. The data presented here, demonstrate distinctly the gradient found from C_3 to C_4 traits. The major findings of this study were that the BS cells dimensions are contrasting to what is discussed in the literature; and three out of four *Homolepis* species are intermediates instead of C_3, and they possess a different level of intermediacy. Both physiological and anatomical results point out that there is a C_3, a Proto-Kranz, 2 C_2 species and the C_4 *M. loliiforme*.

One of the *Homolepis* species, *H. aturensis,* has already been studied and confirmed to be a C_2 species (Khoshravesh et al., 2016). Hence, with this information it is convenient to compare *H. aturensis* results with the other *Homolepis* species. Usually, the main difference between an intermediate and a C_3 species is the activation of the BS cells and a higher concentration of organelles, chloroplasts, mitochondria and peroxisome in the BS cells of intermediate species. Additionally, in C_3 species the compensation point is higher than in other species (Monson & Moore, 1989).

Beginning with *H. glutinosa*, its traits are common to C_3 species. The Γ result is the highest and the results for anatomical measures are distant from *H. aturensis* results, and in opposite direction to those found for the C_4 *M. loliiforme* (FIGURE 1 and 4). On the other hand, the Γ of *H. isocalycia* is between the values found for C_3 and C_2 species, and its anatomical traits, such as IVD, number of M cells between veins, BS cells dimensions and more mitochondria are localized adjacent to the vascular tissue, but more associated to the results found for *H. glutinosa* than the ones found for *H. aturensis* (FIGURE 1, 4 and 6). A lower Γ means that might exist in the BS cells a concentration of CO_2 released from the decarboxylation of glycine, and then the refixation of this photorespiratory CO_2 in the BS cells will occur (Khoshravesh et al., 2016).

A intermediate key step in the C_4 evolution is the enrichment and arrangement of organelles and enzymes in the BS cells. In C_4 species chloroplasts and mitochondria are well arranged to optimize the flux of CO_2 and metabolites, and in intermediate species the organelles are centripetally located in the BS to facilitate the refixation of CO_2. Along the process of transition

from C_3 to intermediates, this early event of BS cells activation characterizes the Proto-Kranz species. They are described as intermediate species that are closer to C_3 features. They have a slight decrease in the Γ values, their BS cells have more organelles than the C_3 BS with BS mitochondria being localized adjacent to the vascular tissue, such as observed for *H. isocalycia* in BS (R. F. Sage et al., 2012, 2014). Therefore, *H. isocalycia* can be classified as a Proto-Kranz species.

Considering the findings for *H. aturensis* in this study, they are similar to the ones found for *H. longispicula*. Both species have related results such as, the same Γ value, a high vein density and number of M cells between veins, lower IVD and a higher concentration of organelles in the BS cells compared to C_3 species. Consequently, *H. longispicula* can be considered a C_2 species. For this species, there is the difference in its leaf thickness, that results in bigger BS cells compared to the ones of *H. aturensis*. In this case *H. longispicula* BS cells dimensions are more similar to the BS cells of *H. glutinosa* (FIGURE 6).

Identifying intermediate species

The carbon isotope data have been used for a while as a technique to identify C_4 species. Nevertheless, the use of this technique is inconsistent as a parameter for differentiation of intermediate species. Once the carbon isotope values of intermediates are quite similar to C_3 values, a result of incomplete compartmentalization of the photosynthetic pathway (Alonso-Cantabrana & von Caemmerer, 2016). The results for carbon isotope of *Homolepis* intermediate species are among the values found for C_3 species (TABLE 2). A different work with *Alloteropsis* species also confirms this pattern, where C_3 like results were observed for intermediate individuals. This shift in carbon isotope towards C_4 values might occur after plants start to fix more CO_2 in the C_4 pathway, especially with the involvement of the enzyme PEPc in the M cells (Lundgren et al., 2016).

With respect to the gas exchange variables, the A/Ci curves, specially Γ results can easily discriminate intermediate species. However, some traits such as A, CE and A/gs can confirm the high efficiency of C_4 species, but cannot discriminate, necessarily, between a C_3 and intermediate species. Intermediate species from *Homolepis* genus have their A, CE and A/gs among C_3 values (TABLE 1).This was also noted for other intermediates species, where the results such as CE were

not high as C_4, instead they were similar to C_3 values (Khoshravesh et al., 2016; Lundgren et al., 2016). Looking at gas exchange variables for *M. loliiforme* the results are statistically different from the other species; especially the gs and consequently A/gs. The gs shows an extremely low value (< 0.2) when compared to the other species. This is explained with the idea of the CCM, where C_4 species with a lower gs are capable to achieve high photosynthetic rates. This value for *M. loliiforme* is even lower than the one observed for the other C_4 species. This can be explained by the fact that *M. loliiforme* plants were collected in their natural environment and was growing for a short period in the greenhouse. Different of the other species, *A. pubescens* that has been grown for a while in the greenhouse condition with plenty of water. This could represent a form of plasticity, an adjustment to the greenhouse environment which is completely different from the natural habitat (TABLE 1).

Another reliable procedure in distinguishing the photosynthetic types is the anatomical measurements, which contribute with a lot of details. With that, a correlation test with Γ and anatomical traits were conducted trying to identify a relationship between those variables (FIGURE 6). The results show a strong and positive correlation, indicating that those anatomical traits can be associated with Γ results and they differ among photosynthetic types in a statistically predictable manner. In this work, as Γ increases the BS cells dimension also increases indicating a C_3 trait, while the opposite is true for C_4 traits; consequently, intermediate values correspond to Proto-Kranz and C_2 species.

Anatomical gradient from C_3 to C_4 traits

Regarding the preconditioned anatomical traits to C_4 evolvement, the number of M cells between veins, interveinal distance (IVD), vein density and ratio of M to BS tissue area (M:BS) the intermediate species results separate them in two groups. One group with *H. longispicula* and *H. aturensis* being similar to C_4 *M. loliiforme* traits; and the other group with *H. glutinosa* and *H. isocalycia* that carries similarities with the C_3 pattern. While morphologically *Homolepis* species look the same, *H. isocalycia* has its anatomical traits closer to C_3 species, *H. aturensis* traits are closer to C_4 species, and *H. longispucula* has similarities with both (FIGURE 4 and S2).

The distance between the two compartments in C_4 species is extremely important to facilitate the transport of metabolites for the C_4 cycle. This is also true for intermediate species during the photorespiratory glycine shuttle. Therefore, during the C_4 pathway evolution several anatomic traits have changed to support this biochemical cycle; especially a low ratio of M to BS tissue (Lundgren et al., 2014; Schlüter & Weber, 2016). The decrease in the ratio of these compartments was influenced by changes in different characters, such as, the number of veins, the number of M cells between veins, the IVD, the area of BS and M cells and others (Lundgren et al., 2014). Beginning with the ratio M:BS itself, the intermediate Proto-Kranz and *H. glutinosa* has the same value, whereas the other two intermediates (C_2) have the same values of *M. loliiforme* (Fig. 4C). Noteworthy, when comparing the *H. glutinosa* ratio M:BS, with other C_3 species, as for example *Apochloa* species that is a near group to *Homolepis*, *H. glutinosa* ratio is closer to C_4 species results than the values found for this other C_3 group (Mendonça et al., 2021). Regarding the values of vein density, as expected, *H. glutinosa* has the lowest value and *M. loliiforme* the highest. The values found for all intermediate species are statistically similar, and they represent values between C_3 and C_4, once again exhibiting the gradient (FIGURE 4A). Considering the space and the number of M cells between veins *M. loliiforme* and *H. longispicula* have the lowest values, while *H. glutinosa* and the Proto-Kranz species have the highest. Higher number of veins and reduced interveinal distance are considered as C_4 features; even though usually C_3 grasses have plenty of veins. This actual works demonstrates that both C_2 species follow the C_4 traits and the Proto-Kranz species is more related to the C_3 pattern. Reinforcing that this condition might be the first step of change to the intermediate state. Besides that, the environment where *H. longispicula* and *M. loliiforme* are found is an open area, with high light intensity and seasonal rainfall. Whereas, *H. glutinosa* and *H. isocalycia* are found in a shadow habitat. This difference, might explain the morphology of these species and its physiological and anatomical traits. Especially, the thicker leaf of *H. longispicula,* which in turns result in a big BS and M tissue area with a high density of veins and a lower IVD.

In C_3 monocots lateral and rank-1 veins are common veins, however rank-2 veins are typical of C_4 species (Sedelnikova et al., 2018). In *Homolepis* species it was seen just rank 1 veins, and the organization of those are quite similar among them. Between the lateral veins there are usually an average number of ran1 veins in both sides, and sometimes one side has different veins. In this organization, for *H. aturensis* it is seen on average 5 rank 1 veins between lateral veins. For *H.*

glutinosa and *H. isocalycia* the average is 4 rank 1 veins, and for *H. longispicula* it decreases for 2 rank 1 veins between lateral veins. Once again, these results are explained by the values of their width, but when looking at the ratios of leaf veins:leaf width it is found that *H. longispicula* has the lowest ratio indicating the highest density of veins per leaf width (Supplementary material TABLE S2). The arrangement of veins, as well as its density will provide a better support to the flux and distribution of photosynthates. More than that, for C_4 and intermediate species the proximity of veins are important for the metabolites shuttle during the process of CO_2 and glycine decarboxylation. Usually, large veins serve as transport of photosynthates to distant areas, whereas small veins serve as transport of metabolites to nearby areas (Ueno et al., 2006). In the literature it is well demonstrated that C_4 species has the higher veins density and a lower IVD than C_3 species. Ueno and coworkers, demonstrate that this difference in density is explained by the number of small longitudinal veins, and the distance between them; instead of a higher number in lateral veins. It is shown that C_4 species has a denser system of small longitudinal veins (rank 1 and 2) and also transverse veins, that connects the longitudinal ones (Ueno et al., 2006). This is in accordance of what it was seem in this actual work, where *M. loliiforme* results show more rank 1 and 2 veins between lateral veins than the other species. Regarding the veins, it is also worth note that for grasses and C_4 species that usually grow in areas with water scarcity, the arrangement of veins is very important for dealing with embolism and cavitation. These features facilitate the flux of metabolites between M and BS cells compartments, being the main function in C_4 cycle, but also improve the water relations under different environmental conditions (Sage et al., 2014). Which can be important for these grasses that are found in arid regions.

Considering the BS cell dimension, another trait that is well studied in the literature of C_4 evolution; it is discussed that because this cell compartment needs to accommodate more organelles, it needs to be large (Lundgren et al., 2014). Most of the studies are done with images from cross sections. However, with them the size of these BS cells can be wrongly estimated, because in the three-dimensional vision the cells are not round but cylindric. So, in the present work longitudinal images were also used from confocal microscopy to have an original idea of BS cells dimensions. The findings in this work have contradictory results compared to what is seem in the literature. The measures of BS cells demonstrate clearly the gradient found from C_3 to C_4 species, however, here the biggest BS cells values were found for C_3 species *H. glutinosa*, and the smallest for C_4 species *M. loliiforme* (FIGURE 5 and 6). With the decrease in cell area, both length

and width decrease, and consequently it will promote a higher number of BS cells along the length of the veins (FIGURE 5). The arguments about the increase of BS cells mention that enlarged cells can accommodate more organelles and increase its engagement on carbon fixation (Sage et al., 2012). Here, the difference might be that, instead of having large amounts of organelles in just one big cell, there are many cells along the veins and also more veins. This different pattern was observed in a recent work where it was demonstrated that C_4 species have a smaller cross-sectional area. (Khoshravesh et al., 2020). For monocots it was also observed that some C_3 clades sister of C_4 clades also have species with large BS cells, although the measurements were done on leaf cross-sections (Griffiths et al., 2013; T. L. Sage et al., 2013). In a different study the BS cells (the outer cells) of C_3 species belonging to the BEP group are smaller compared to C_3 species from PACMAD (Christin et al., 2013). Many times, this observed difference might be explained by the method that it was used, however other times this difference might exist among different species and groups. In a different study about plasmodesmata in grasses, using the confocal microscopy looking at a 3-D geometry of the cells, the authors also observed that in fact C_4 species does not have necessarily bigger BS cells. Instead, the total area of BS per leaf area might be bigger in C_4 species (Danila et al., 2018). Previously, Brown have seemed in paradermal observations that the size of BS cells of Kranz species (C_4) were not bigger than the ones in non-Kranz species (C_3). In his work, it was recorded the L:W ratio of the cells but not the area or volume. The results demonstrate a variety in ratios found among species, and the bigger numbers (≥ 2.5) were found for non-Kranz species, while for Kranz species it was recorded ratios of 0.85. Besides that, he demonstrates the variety among different groups as well (Brown, 1974). In the actual work, the average of ratio found for C_3 species is 2.3 and 1.7 for C_4 species. Therefore, more studies on this trait and with this confocal technique is necessary to solve this question. Is it a difference found between monocots and eudicots? Or this is a difference found between the chosen techniques?

What is used to explain the size of BS cells for C_4 plants can also explain the big BS cells found in C_3 and intermediate species (Lundgren et al., 2019). C_4 plants have more veins that can help to deal with water scarcity, while C_3 and intermediate species have less veins than C_4, but bigger BS cells. These big cells may provide advantage on their environment, specially under drought conditions, serving as water reservoirs (Griffiths et al., 2013). The migration of C_3 species, from humid and close environment to open and dry areas, results in plants dealing with environmental issues. With that the process of changes and adaptations will keep these plants alive.

Because of that, in the same severe area where are also found a variety of C_4 species it might be observed C_3 and intermediates species with such anatomical and morphological alterations (Osborne & Sack, 2012).

PERSPECTIVES

The photosynthetic capacity of the plants has a great importance for their growth, survival and reproduction. Regarding to crop species, its productivity will be hence directly correlated with its photosynthetic activity. Considering that, the current environmental conditions, due to climate change, are quite similar to those events when C_4 evolved, except that CO_2 has been arising. Then, the traits that were acquired by intermediate and C_4 plants might be advantageous in the present scenario with such climate alteration. The changes observed along the process of the C_4 evolvement lead to an establishment of species adapted to environments with higher temperature, light irradiance and lower water availability (Bräutigam et al., 2018; Osborne & Sack, 2012). The findings of different intermediate species, especially in the monocot group is extremely important to address more species to be studied for C_4 evolution, and to find out which traits are present along the evolvement. Hence, understanding how they evolved, especially the different traits observed in intermediates species, can be helpful to modify C_3 species towards the enhancement of its fitness in such climate change acclimation. Additionally, this type of information contributes greatly with a broad of agronomical and botanical studies and especially with the literature of C_4 evolution.

CONCLUSION

With the data presented here it we used anatomical and photosynthetic physiology data to characterize the photosynthetic types in the genus *Homolepis* and the species in the sister group, *M. loliiforme*. We identified intermediate species in the genus *Homolepis.* There is a C_3 – *H. glutinosa*, a Proto-Kranz – *H. isocalycia,* and two C_2 – *H. aturensis* and *H. longispicula.* It was also described the characteristics of the C_4 *M. loliiforme.* The data illustrate the gradient transition in the anatomical and physiological traits from C_3 to C_4 observed for all species. Moreover, the results show a new perception, it was found out that the C_3 grasses and intermediates species in this work have bigger BS cells dimensions than the C_4 species. Despite few works have demonstrate

that with longitudinal view this parameter could be different the great majority of works show the opposite direction, C_4 with bigger cells.

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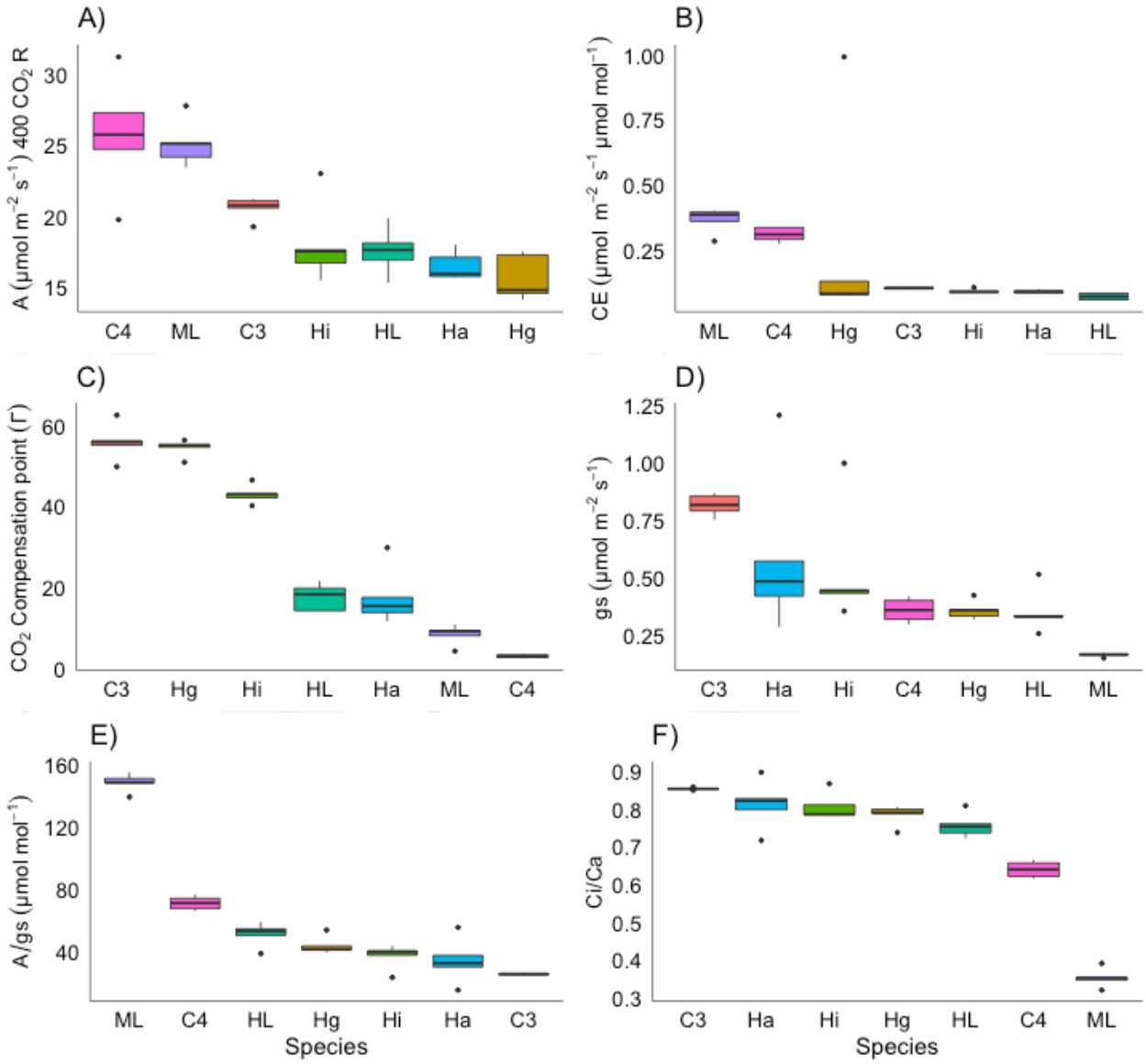
SUPPLEMENTARY MATERIAL

Table S1- Field coordinates of species collection.

Species	Localization
Homolepis glutinosa (Sw.) Zuloaga & Soderstr.	21º 19’ 58” S / 44º 58’ 37” W
Homolepis isocalycia (G.Mey.) Chase	19° 12' 29" S / 43° 29' 58” W
Homolepis longispicula (Döll) Chase	19º 17’ 11” S / 43º 35’ 18” W
Mesosetum loliiforme (Hochst.) Chase	19º 17’ 12” S / 43º 35’ 22” W
Homolepis aturensis Chase	9°39’27.4”N 82°45’08.0”W

Source: From author (2022).

Figure S1- Distribution of the leaf gas exchange summary results for four *Homolepis* species, *M. loliiforme* and two other grasses (*Phragmites australis* and *Anthephora pubescens*) C_3 and C_4 reference. A) CO_2 assimilation; B) Rubisco carboxylation efficiency; C) CO_2 compensation point; D) Stomata conductance; E) Intrinsic water use efficiency and F) Ratio of intercellular and ambient CO_2 concentration. The boxplot indicates the medians, n= 3 for *M. loliiforme*, *P. australis* and *A. pubescens*, and n= 5 for other species.

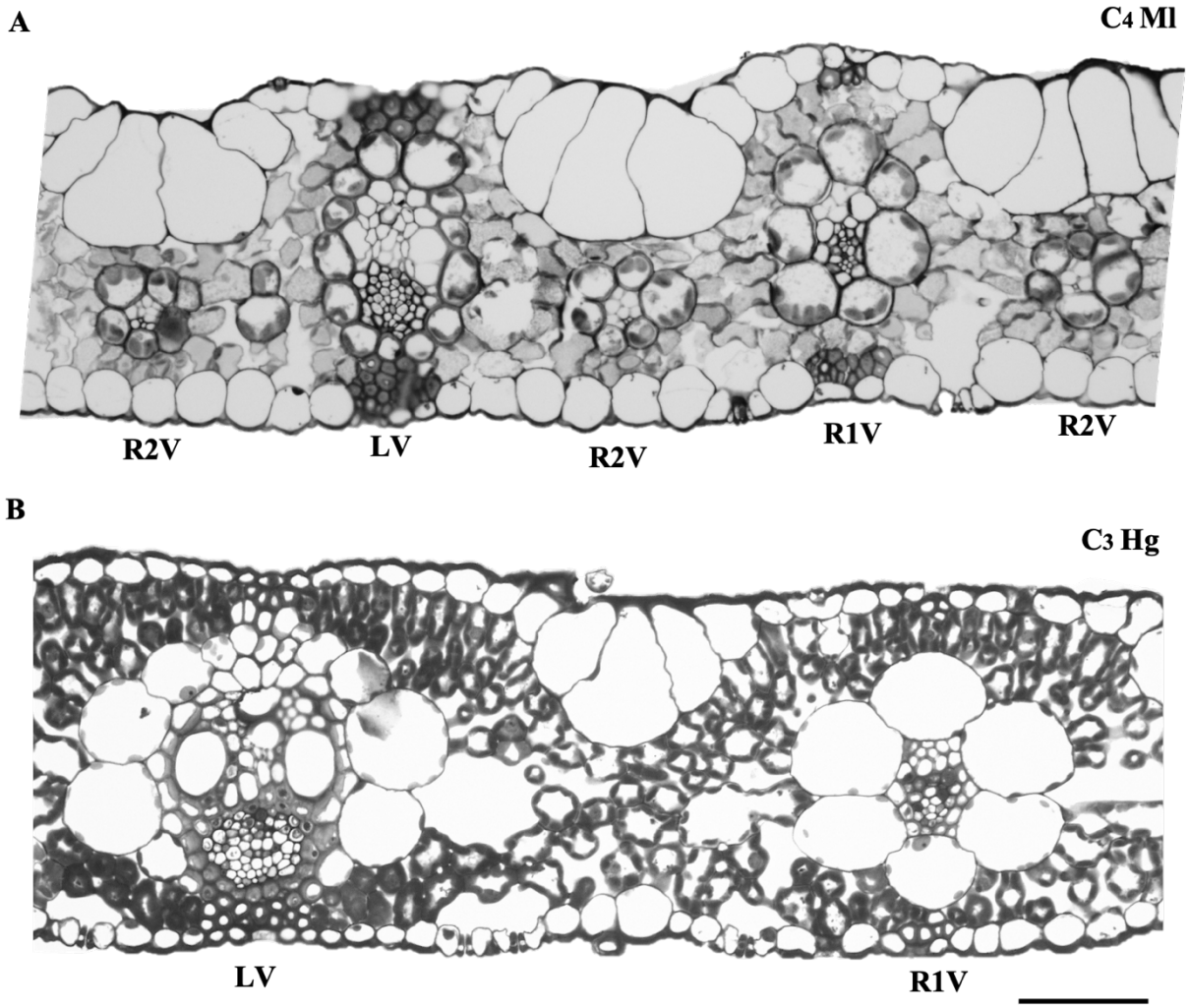


Source: From author (2022).

Figure S2- Leaves of *Homolepis* species and *Mesosetum loliiforme*. Hg – *H. glutinosa*, Hi – *H. isocalycia*, Ha – *H. aturensis*, Hl – *H. longispicula* and Ml – *M. loliiforme*. Scale bar = 5cm.

Source: From author (2022).

Figure S3- Identification of lateral and rank 1 and 2 veins in A) C_4 Ml - *M. loliiforme* and B) C_3 Hg – *H. glutinosa.* LV – lateral vein, R1V – rank 1 vein, R2V – rank 2 vein. Scale bar = 50 µm.



Source: From author (2022).

Table S2 – Identification of the number of veins in the entire leaf of *Homolepis* species and *M. loliiforme*. A) Rank 1 veins are observed in all species, rank 2 veins are found only in C_4 species and they were summed with rank 1 veins. B) Lateral veins. Hg – *H. glutinosa*, Hi – *H. isocalycia*, Ha – *H. aturensis*, Hl – *H. longispicula* and Ml – *M. loliiforme*. # - number. L – lateral veins. R1v – Rank 1veins. Right and Left correspond to the sides dividing the leaf in the middle.

A

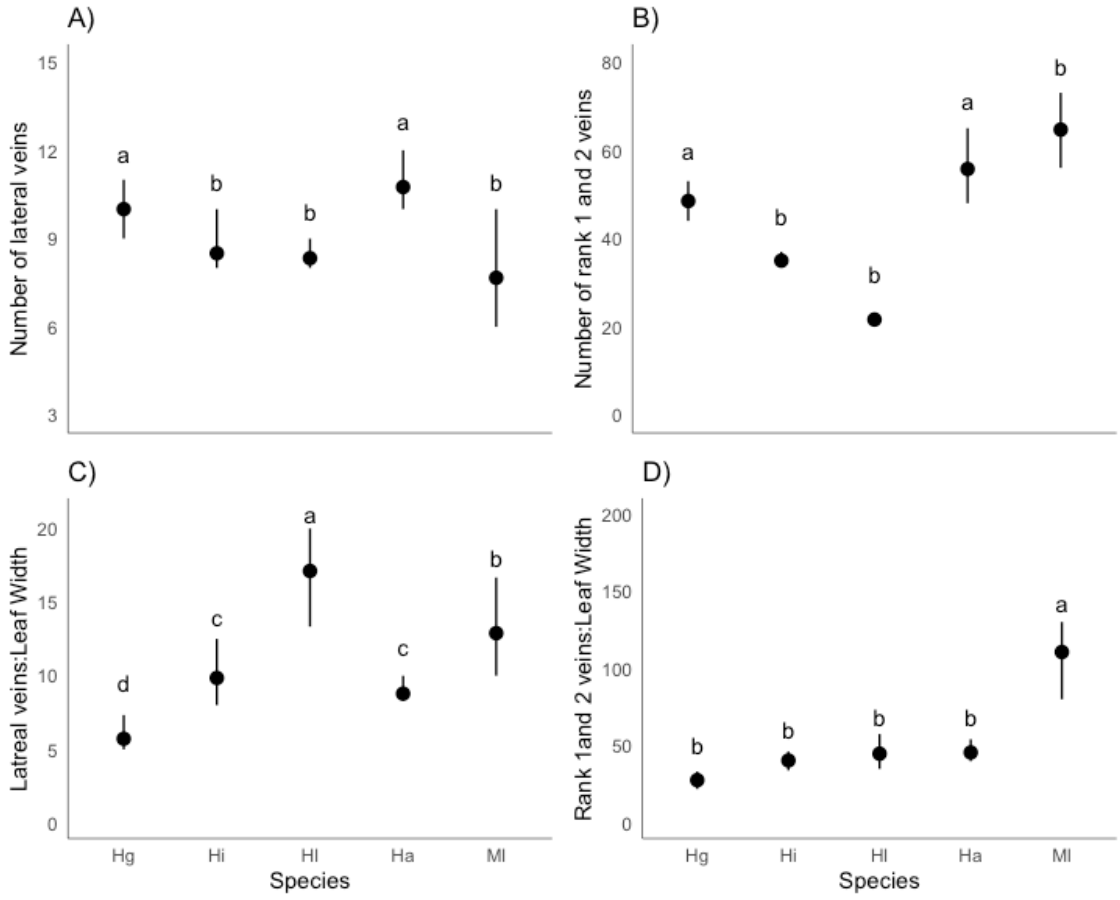
Species	# R1v - from midvein to L1 -Right	# R1v- from midvein to L1 -Left	# R1v between L1 & L2 - Right	# R1v between L1 & L2 - Left	# R1v between L2 & L3 - Right	# R1v between L2 & L3 - Left	# R1v between L2 & leaf margin - Right	# R1v between L2 & leaf margin - Left	Total of R1v
Hg	4	3	4	4	5	4	18	16	49
Hi	4	4	5	4	4	3	10	9	35
Hl	2	2	2	2	3	3	7	6	22
Ha	5	4	6	5	6	5	20	16	56
Ml	9	9	7	7	7	7	17	16	65

B

Species	# Lv -Right	# Lv - Left	Total of Lv	Leaf width
Hg	5	5	10	1.8
Hi	4	4	9	0.9
Hl	4	4	8	0.5
Ha	6	5	11	1.2
Ml	4	3	8	0.6

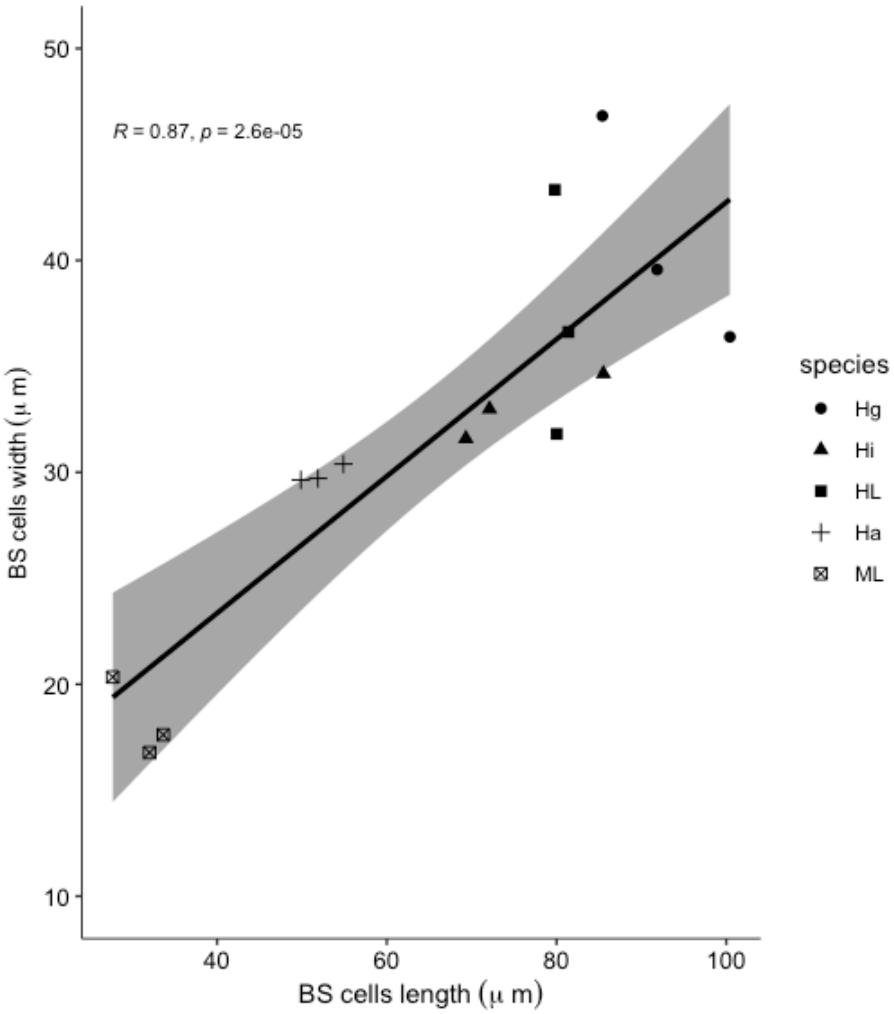
Source: From author (2022).

Figure S4- Determination of leaf veins number (lateral and rank 1 and 2 veins) and ratio of veins: leaf width in *Homolepis* species and *M.loliiforme*. A) Number of lateral veins; B) Number of rank 1 and 2 veins; C) ratio of lateral veins and leaf width and D) Ratio of rank 1 and 2 veins and leaf width. Points indicate the mean with min and max; n=4. Letters indicate common statistical groups according to one-way ANOVA followed by a Scot-Knots's post-hoc test ($P \leq 0.05$). Hg – *H. glutinosa*, Hi – *H. isocalycia*, Ha – *H. aturensis*, Hl – *H. longispicula* and Ml – *M. loliiforme*.



Source: From author (2022).

Figure S5- Correlation between BS cells length and width (μm). Method of Pearson correlation. Hg – *H. glutinosa*, Hi – *H. isocalycia*, Ha – *H. aturensis*, Hl – *H. longispicula* and Ml – *M. loliiforme*.



Source: From author (2022).