ASPECTOS MORFOLÓGICOS, FUNCIONAIS E EVOLUTIVOS DA BAINHA DO FEIXE EM FOLHAS HOMOBÁRICAS E HETEROBÁRICAS DE LEGUMINOSAS

FERNANDA HELENA PALERMO

Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Doutora no Programa de Pós-Graduação em Ciências Biológicas (Botânica), Área de concentração: Morfologia e Diversidade Vegetal.

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“Luz do sol
Que a folha traga e traduz
Em verde novo
Em folha, em graça, em vida, em força, em luz...”

Caetano Veloso

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Resumo

Os feixes vasculares imersos no mesofilo das folhas são rodeados por células que formam a bainha dos feixes. Em diversas espécies vegetais, essa bainha pode se projetar em direção à epiderme de ambos os lados do limbo foliar constituindo extensões da bainha dos feixes (EBF). Folhas que apresentam o mesofilo compartimentalizado pela presença de EBF são denominadas heterobáricas, enquanto que folhas desprovidas de EBF são classificadas como homobáricas. Embora a presença/ausência de EBFs seja um caráter constitutivo entre as espécies, fatores climáticos parecem afetar o desenvolvimento dessas estruturas. A compartimentalização do mesofilo pela EBF está associada a diferenças nos aspectos funcionais das folhas heterobáricas e homobáricas, o que pode estar relacionado a peculiaridades ultraestruturais das células da bainha dos feixes. Diferenças nos aspectos funcionais entre espécies com folhas homobáricas e heterobáricas como condução de água, luminosidade e gases, tem sido relacionado ao sucesso da colonização de diferentes ambientes pelas plantas. Este trabalho teve como objetivo caracterizar a ultraestrutura das células da bainha do feixe em folhas homobáricas e heterobáricas e investigar a influência da luz e disponibilidade hídrica no desenvolvimento e funcionamento das EBF, além de elucidar como se deu a evolução dessa característica anatômica dentro de um Clado de Leguminosae. Indivíduos de *Machaerium acutifolium* Vogel que apresentam folhas heterobáricas e de *Dimorphandra mollis* Benth que possuem folhas homobáricas foram submetidos a diferentes condições de luz e disponibilidade hídrica em casa de vegetação. Folhas formadas após a aplicação dos tratamentos foram processadas para análises ao microscópio de luz e a distância entre as bainhas dos feixes foi medida; além disso, os índices de trocas gasosas foram mensurados com IRGA. Amostras do limbo foliar de ambas as espécies ocorrentes em ambiente natural do cerrado foram processadas para análises ao microscópio eletrônico de transmissão. Folhas de 75 espécies de *Adesmia* DC., *Amicia* Kunth, *Nissolia* Jacq., *Poiretia* Vent. e *Zornia* J.F. Gmel., foram obtidas de herbários nacionais e internacionais e processadas para estudos anatômicos a fim de se obter o mapeamento da distribuição de espécies com folhas homobáricas e heterobáricas no clado Adesmia e buscar associações entre o hábito de vida e área de ocorrência dessas plantas e o tipo foliar. A distância entre as bainhas dos feixes não variou entre as duas espécies mantidas sob diferentes condições de luz e disponibilidade hídrica. *M. acutifolium* apresentou menores taxas de assimilação, transpiração e condutância estomática e maiores valores de eficiência do uso da água em comparação com *D. mollis*, independente do tratamento a que foram submetidas. Esses resultados podem indicar que as EBF podem estar associadas a estratégias inerentes das espécies com folhas homobáricas e heterobáricas para uso de água e luz. Ultraestruturalmente, as células da bainha do feixe em *D. mollis* apresentaram campos de pontoações primárias com plasmodesmos bastante amplos indicando que a rota simplástica de transporte de água e solutos entre as nervuras e o mesofilo parece ser favorecida nessa espécie. As células da bainha do feixe de *M. acutifolium* mostraram menor abundância de plastídios com tamanho menor e tilacóides menos desenvolvidos em comparação com *D. molli*s, o que corrobora a ideia de que as células das EBF podem facilitar a penetração da luz no mesofilo por serem mais translúcidas. A análise da distribuição de espécies com diferentes tipos foliares no clado Adesmia mostrou que a presença/ausência de EBF parece estar relacionada ao hábito de vida e ao grupo taxonômico; nossos dados sugerem que esse caráter parece ter evoluído de folhas heterobáricas para homobáricas no grupo estudado. Os resultados obtidos mostram novas perspectivas para o estudo do desenvolvimento e funcionamento das extensões de bainha do feixe, gerando informações sobre os aspectos ecológicos, fisiológicos e evolutivos das espécies estudadas.

Palavras-chave: evolução, extensão de bainha dos feixes, Leguminosae, trocas gasosas, ultraestrutura

Abstract

The vascular bundles immersed in the leaf mesophyll are surrounded by cells that form the bundle sheath. In several plant species, this sheath can project towards the epidermis on both sides of the leaf blade, constituting bundle sheath extensions (BSE). Leaves that present the mesophyll compartmentalized by BSE are named heterobaric, while leaves without BSE are classified as homobaric. Although the presence/absence of BSE is a constitutive feature among species, climatic factors seem to affect the development of these structures. The compartmentalization of the mesophyll by BSE is associated with differences in the functional aspects of heterobaric and homobaric leaves, which may be related to ultrastructural peculiarities of the bundle sheath cells. Differences in functional aspects between species with homobaric and heterobaric leaves, such as conduction of water, luminosity, and gases, have been related to the successful colonization of different environments by the plants. This work aimed to characterize the ultrastructure of the bundle sheath cells in homobaric and heterobaric leaves and to investigate the influence of light and water availability on the development and functioning of the BSE, in addition to elucidating how this anatomical feature evolved within a clade of Leguminosae. Individuals of *Machaerium acutifolium* Vogel that presents heterobaric leaves and *Dimorphandra mollis* Benth that have homobaric leaves were submitted to different conditions of light and water availability in a greenhouse. Leaves formed after the treatments were processed for analysis under light microscopy and the distance between the bundle sheath cells was measured; gas exchange rates were measured with IRGA. Leaf blade samples of both species occurring in the natural environment of the Cerrado were processed for analysis under transmission electron microscopy. Leaves of 75 species of Adesmia DC., *Amicia* Kunth, *Nissolia* Jacq., *Poiretia* Vent. and *Zornia* J.F. Gmel., were obtained from worldwide herbaria and processed for anatomical studies to map the distribution of species with homobaric and heterobaric leaves in the Adesmia clade and to seek associations between the life habit and area of occurrence of these plants and the leaf type. The distance between the bundle sheaths did not vary in the two species maintained under different conditions of light and water availability. *M. acutifolium* presented lower rates of assimilation, transpiration, and stomatal conductance and higher values of water use efficiency compared to *D. mollis*, regardless of the treatment to which they were submitted. These results may indicate that BSE may be associated with inherent strategies of species with homobaric and heterobaric leaves for water and light use. Ultrastructurally, the bundle sheath cells in *D. mollis* presented primary pit fields with very large plasmodesmata indicating that the symplastic route of water and solutes transport between the veins and the mesophyll seems to be favored in this species. The bundle sheath cells of *M. acutifolium* showed a lower abundance of cloroplasts with smaller size and less developed thylakoids compared to *D. mollis*, which corroborates the idea that BSE cells can facilitate the penetration of light into the mesophyll because these cells are more translucent. The analysis of the distribution of species with different leaf types in the Adesmia clade showed that the presence/absence of BSE seems to be related to life habits and taxonomic group; our data suggest that this character seems to have evolved from heterobaric to homobaric leaves in the studied group. The results obtained show new perspectives for the study of the development and functioning of the bundle sheath extensions, generating information about the ecological, physiological, and evolutionary aspects of the studied species.

Keywords: bundle sheath extensions, evolution, gas exchange, Leguminosae, ultrastructure

INTRODUÇÃO GERAL

As folhas são órgãos especializados na realização da fotossíntese e podem apresentar grande plasticidade morfológica de acordo com o ambiente em que ocorrem (Tsukaya 2018), além de variações estruturais e funcionais entre os diferentes grupos taxonômicos de plantas (Dickison 2000).

O limbo foliar é formado por três sistemas de tecidos: o sistema dérmico, responsável pelo revestimento e que se origina a partir da protoderme; o sistema fundamental que constitui os tecidos presentes no mesofilo e córtex da nervura principal e que tem sua origem do meristema fundamental; e sistema vascular, originado do procâmbio e que constituem as nervuras de diferentes calibres (Esau 1977; Menezes *et al.* 2006).

A vascularização das folhas se estende desde o caule, de forma contínua, passando pelo pecíolo e se ramificando na lâmina foliar (Esau 1977; Roth-Nebelsick 2001) num arranjo específico de acordo com o táxon (Dickison 2001). De forma geral, a venação nas Eudicotiledôneas é composta por uma nervura principal, de maior calibre, que se estende pela lâmina foliar e se ramifica em nervuras de menor calibre, formando uma rede (Menezes *et al.* 2006). Essas nervuras podem ser classificadas de acordo com seu calibre (Roth-Nebelsick 2001). As nervuras principais são também chamadas de nervuras primárias, e a partir delas se ramificam as nervuras secundárias; da mesma forma, as nervuras terciárias se originam das secundárias e assim por diante.

Na maioria das plantas, as nervuras apresentam xilema voltado para a face adaxial da folha e floema voltado para a face abaxial do limbo, constituindo feixes vasculares colaterais (Dickison 2000). Envolvendo as nervuras, ocorre uma camada de células especializadas originadas do meristema fundamental, denominada bainha do feixe (Dickison 2000) ou endoderme (Menezes 1971; Menezes et al. 2006). As células da bainha do feixe podem apresentar grãos de amido em seu interior e estrias de Caspary em suas paredes e atuam na comunicação entre os tecidos vasculares e as células do parênquima clorofiliano (Menezes *et al.* 2006), desempenhando um papel importante no funcionamento das folhas (Dickison 2000). Em determinadas espécies vegetais, essa bainha constituída por células incolores pode se estender até a epiderme de uma ou de ambas as faces do limbo foliar conectando os feixes vasculares com a epiderme (Esau 1977). Em algumas plantas, fibras originadas do periciclo podem acompanhar a extensão da bainha do feixe até a epiderme (Menezes et al. 2006). Tais colunas verticais de células foram descritas como extensões de nervuras (“vein-extensions”) ou linhas de células (Richter 1898, *apud* Terashima 1992), tendo sido mais tarde denominadas extensões de bainha dos feixes (Wylie 1952).

Classificação dos tipos foliares quanto às extensões de bainha dos feixes

As folhas das angiospermas podem ser classificadas como heterobáricas ou homobáricas, conforme a presença ou ausência de extensões de bainha do feixe (EBF) ao redor das nervuras foliares, respectivamente. Nas folhas heterobáricas, as células da bainha que envolve os feixes vasculares se estendem até a epiderme de ambas as faces do limbo foliar. As EBF são comumente encontradas ao redor de nervuras de menor calibre, mas também podem estar presentes ao redor de nervuras maiores (Wylie, 1952). Nesse tipo foliar, o mesofilo fica dividido em pequenos compartimentos que restringem a circulação lateral de gases, fazendo com que os índices de trocas gasosas se mantenham independentes em cada compartimento (Pieruschka et al. 2005; Buckley et al. 2011).

No início do século XX, esses compartimentos formados pela presença das EBF foram nomeados como câmaras de ar (Neger 1912, 1918 *apud* Terashima 1992). Posteriormente, Raschke et al. (1990) se referiram a esses compartimentos como alvéolos, numa alusão às pequenas cavidades ou sacos de ar do pulmão. Alguns autores também utilizaram o termo areólas em referência a tais compartimentos (ver Terashima 1992). No entanto, o termo aréolas também pode ser utilizado para se referir às áreas formadas pelas intersecções de veias que não necessariamente possuem EBF (Esau 1977, Roth-Nebelsick 2001), o que pode gerar confusão nomenclatural. Atualmente, o nome mais aceito para essa estrutura é compartimento de extensão de bainha do feixe, cunhado no trabalho de revisão de Terashima (1992).

Os termos homobárica e heterobárica tem sua origem no grego, onde: *homo* significa semelhante, *hetero* significa diferente e *bárico* se refere à pressão. Assim, as expressões homobárica e heterobárica fazem referência a pressão de CO_2 dentro do mesofilo foliar. Isso porque nas folhas heterobáricas, a pressão de CO_2 pode variar entre os compartimentos formados pelas EBF de forma mais acentuada do que nas folhas homobáricas, devido ao número e a abertura diferencial de estômatos em cada compartimento (Terashima 1992).

As folhas heterobáricas típicas são aquelas que possuem EBF nas nervuras de segunda, terceira e, eventualmente, quarta ordem (Wylie 1952, Terashima 1992), enquanto as folhas homobáricas não apresentam EBF em nenhuma nervura do mesofilo foliar. No entanto, alguns autores também consideram aquelas folhas com EBF apenas nas nervuras de segunda ordem como heterobáricas (Terashima 1992). Num estudo recente, Rodrigues et al. (2017) propuseram nomenclatura específica para as variações apresentadas pelas folhas quanto às EBF, tendo sido descritos quatro tipos foliares: folhas heterobáricas típicas (com EBF nas nervuras de segunda e terceira ordens e eventualmente também em nervuras menores); folhas homobáricas (desprovidas de EBF); folhas heterobáricas amplas (com EBF apenas nas nervuras de segunda ordem levando a divisão do mesofilo em grandes compartimentos) e folhas semi-heterobáricas (com EBF que se estendem apenas em direção a uma das faces do limbo foliar levando a uma compartimentalização incompleta do mesofilo). Nesse mesmo estudo, os autores mostraram que folhas heterobáricas amplas apresentam características de trocas gasosas semelhantes às heterobáricas típicas, enquanto as semi-heterobáricas apresentam taxas fotossintéticas semelhantes às folhas homobáricas.

Aspectos ultraestruturais da bainha dos feixes

As células da bainha dos feixes podem apresentar peculiaridades estruturais de acordo com os grupos de plantas onde ocorrem (Rogowski et al. 2019, Xiu et al. 2020). Estudos demonstram que os aspectos subcelulares das células da bainha dos feixes podem estar diretamente relacionados ao funcionamento dessas células e da folha como um todo (Xiu et al. 2020). Peculiaridades ultraestruturais são conhecidas principalmente quanto ao desenvolvimento das estruturas de comunicação simplástica e a organização e morfologia dos cloroplastos nas células de bainha do feixe em espécies com diferentes características anatômicas e fisiológicas.

Em folhas de plantas com metabolismo fotossintético C4, as células da bainha dos feixes são caracterizadas pela presença de plastídios com grana bem estruturado, o que não ocorre nas células da bainha dos feixes de plantas C3 (Robinson-Berrs & Evert 1991, Taniguchi et al. 2003, Omoto et al. 2009, Rogowski et al. 2019). Nas espécies vegetais com metabolismo C4, as células da bainha dos feixes apresentam grãos de amido volumosos nos cloroplastos (Robinson-Berrs & Evert 1991, Ajao et al. 2017). Ainda, em tais espécies com metabolismo C4, estudos tem reportado a ocorrência de plasmodesmos amplos garantindo a comunicação entre células do mesofilo e da bainha dos feixes (ver Dickinson 2000, Robinson-Berrs & Evert 1991). Considerando as diferenças fisiológicas registradas em literatura para folhas homobáricas e heterobáricas (Rodrigues et al. 2017, Kenzo et al. 2007, Nikolopoulus 2002, Pieruschka et al. 2005, Inoue et al. 2015), aspectos distintivos nas características ultraestruturais das células da bainha dos feixes entre os dois tipos foliares são esperados. Entretanto, estudos comparativos entre os aspectos subcelulares da bainha dos feixes em folhas homobáricas e heterobáricas não foram encontrados em literatura.

Aspectos funcionais de folhas homobáricas e heterobáricas

A presença das EBF em folhas heterobáricas parece estar associada às diferenças em aspectos fisiológicos em comparação com as folhas homobáricas. Com relação a atuação das EBF no transporte de água na folha, estudos têm apontado para maiores valores de uso eficiente da água (*EUA*) em plantas com folhas heterobáricas, o que estaria relacionado com menores taxas de transpiração (Liakoura et al. 2009). Ainda, o menor consumo de água em espécies com folhas heterobáricas seria resultante do transporte de água de forma mais eficiente nessas folhas (Inoue et al. 2015), o que estaria relacionado à menor resistência hidráulica no limbo foliar devido à presença de EBF (Buckley et al. 2011) que representaria um caminho mais eficiente para o fluxo de água no mesofilo (Wylie 1952). Além disso, tem sido demonstrado que as EBF também podem atuar como barreiras a difusão de gases no mesofilo foliar, fazendo com que a concentração de gases dentro de cada compartimento do mesofilo seja maior auxiliando na manutenção da fotossíntese em espécies com folhas heterobáricas (Pieruschka *et al.* 2005).

As EBF podem ser compostas por uma ou mais camadas de células parenquimáticas e/ou esclerenquimáticas. Quando compostas por parênquima, as EBF apresentam células ovaladas ou alongadas com paredes delgadas (Wylie 1952) e sem espaços intercelulares (Terashima 1992, Metcalfe & Chalk 1979). Outra característica marcante é a ausência ou escassez de cloroplastos nessas células (Wylie 1952, Ueno & Hatakeyama 2018), que dão a elas o aspecto translúcido quando observadas ao microscópio de luz. Estudos sugerem que as células translúcidas das EBF seriam responsáveis por permitir a entrada de luz para as camadas mais internas do mesofilo, sobretudo em folhas mais espessas (Nikolopoulous et al. 2002), funcionando como "janelas" (Karabourniotis et al. 2000) para o mesofilo.

Por outro lado, folhas homobáricas parecem apresentar parênquima esponjoso mais desenvolvido (Vogelmann et al. 1996, Boeger et al. 2016) o que facilitaria a captação de luz difusa nessas espécies (Terashima 1992). Além disso, a ausência de EBF permitiria maior difusão lateral de gases no mesofilo foliar (Kenzo et al. 2007, Liakoura et al. 2009). Essa característica pode ser vantajosa, sobretudo em situações de estresse, pois mesmo com os estômatos fechados, a maior circulação de gases auxiliaria na manutenção das taxas fotossintéticas (Pieruschka et al. 2005). Nesse sentido, fica claro que a presença ou ausência das EBF pode conferir diferentes estratégias fisiológicas para as espécies, dependendo dos fatores abióticos do ambiente em que elas ocorrem.

Distribuição de espécies com folhas homobáricas e heterobáricas

A distribuição de espécies com diferentes tipos foliares parece estar associada com as características do ambiente e parece conferir vantagens em termos estruturais e funcionais em determinadas condições ambientais. Em plantas de ambientes xéricos, por exemplo, as EBF atuariam na movimentação da água no mesofilo, além de conferir proteção mecânica aos feixes vasculares (Aoki et al. 1978, Van der Merwe et al. 1994, Evert 2013, Boeger et al. 2016). Em situações de estresse hídrico, a presença de EBF pode auxiliar na sustentação mecânica das folhas, evitando o colapso do mesofilo (Aoki et al. 1978, Boeger et al. 2016).

Espécies com folhas homobáricas podem ser encontradas em maior abundância em ambientes quentes e úmidos, como as florestas tropicais (Kashimura et al. 2000, Terashima 1992, Pieruschka et al. 2005), enquanto espécies com folhas heterobáricas são mais comuns em ambientes quentes e secos, como as savanas e desertos (Kenzo et al. 2007). Nesse contexto, num estudo inédito envolvendo plantas do cerrado, Mendes et al. (2016) estudaram a distribuição de 131 espécies com folhas homobáricas ou heterobáricas pertencentes a 54 famílias ocorrentes no cerrado *sensu stricto*, no cerradão e na floresta estacional semidecidual. Do total das espécies heterobáricas registradas, 91,2% ocorreram no cerrado, o que, segundo os autores, pode estar associado às condições do ambiente, como maiores taxas de luminosidade. Ainda nesse estudo, Mendes et al. (2016) não encontraram correlação entre os tipos foliares e o hábito das plantas estudadas.

Por outro lado, Boeger et al. (2016), num estudo envolvendo espécies de Mata atlântica sugeriram que a distribuição das espécies com diferentes tipos foliares seria independente do ambiente, mas estaria relacionada com o estrato vegetacional que essas plantas ocupam e seria resultante da estratificação da luz dentro dos ambientes. Assim, estudos sugerem que espécies de dossel, emergentes e de clareira, que tem suas folhas expostas a altas taxas de radiação solar e baixa umidade do ar, apresentariam folhas heterobáricas (Kenzo et al. 2007, Boeger et al. 2016, Inoue et al. 2015), ao passo que as espécies com folhas homobáricas seriam mais abundantes nos estratos mais baixos da vegetação (Boeger et al. 2016, Kenzo et al. 2007).

A relação do tipo foliar quanto à presença/ausência de EBF com os grupos taxonômicos tem sido apontada (Kenzo et al. 2017; Mendes et al. 2016). Alguns gêneros botânicos apresentam homogeneidade com relação ao tipo foliar, independente do ambiente ou estrato vegetal em que as espécies ocorrem (Mendes et al. 2016; Boeger et al. 2016); por outro lado, podem ocorrer espécies congêneres com folhas homobáricas e heterobáricas (Kenzo et al. 2007). Nesse sentido, o comportamento evolutivo das EBF nos diferentes grupos vegetais é um aspecto que merece atenção. No entanto, até o momento, não foram encontrados estudos sobre a evolução desse caráter em grandes grupos vegetais.

Influência de fatores ambientais no desenvolvimento das extensões de bainha do feixe

Embora a presença ou ausência de EBF seja uma característica constitutiva das espécies vegetais, alguns fatores ambientais parecem influenciar em seu desenvolvimento (Wylie 1951; Lynch et al. 2012). Wylie (1951) mostrou que espécies arbóreas de clima temperado com folhas heterobáricas apresentaram aumento no espaçamento entre as EBF quando em condições de sombra; no entanto, o autor realizou medidas apenas em três folhas de cada espécie. Em outro estudo com espécies arbóreas de ambiente temperado, Lynch et al. (2012) mostraram que o aumento da radiação solar e a redução da precipitação podem causar diminuição no espaçamento das EBF no limbo das folhas heterobáricas. Visto que a estrutura de venação está intimamente ligada ao desempenho fisiológico das espécies (Roth-Nebelsick et al. 2001), esse resultado pode indicar que as EBF estão envolvidas na adaptação foliar a variações nas condições microambientais. Para espécies de clima tropical não foram encontrados estudos nesse contexto.

O Clado Adesmia (Leguminosae, Papilionoideae)

O Clado Adesmia, pertencente à Família Leguminosae, subfamília Papilionoideae, é constituído por cinco gêneros (*Adesmia* DC., *Amicia* Kunth, *Nissolia* Jacq., *Poiretia* Vent. e *Zornia* J.F. Gmel) e mais de 360 espécies com diferentes hábitos vegetativos e que estão distribuídas nas regiões pantropical e neotropical (Klitgaard & Lavin 2005).

Adesmia, Amicia, Nissolia e *Poiretia* possuem distribuição nas Américas enquanto *Zornia* apresenta distribuição mais ampla, com representantes na América, África, Oceania e Ásia (Mohlenbrock 1961). *Adesmia* possui cerca de 240 espécies distribuídas na região dos Andes e no sudeste da América do sul (Burkart 1967, Lavin et al. 2001, Ignaci et al. 2013) e seus representantes possuem hábito herbáceo ou arbustivo. *Amicia* é o gênero com menor número de representantes do Clado Adesmia, apresentando seis espécies de ervas e arbustos que ocorrem no México e na região Andina da América do Sul (Rudd 1981, Sarkinen & Hugues 2015). O gênero *Nissolia,* que recentemente foi sinonimizado com *Chaetocalyx* (Moura et al. 2018), apresenta 29 espécies com hábito trepador (Rudd 1956, Rudd 1972a, Rudd 1975, Rudd 1991, Moura et al. 2018) endêmicas das Américas, ocorrendo desde os Estados Unidos até o Uruguai (Moura et al. 2018) com centros de diversidade no México e Brasil. *Poiretia* apresenta 12 espécies de arbustos, subarbustos e trepadeiras com ampla distribuição nas Américas (Rudd 1972b), especialmente no Brasil (Müller 1984, Mendes et al. 2019). *Zornia* apresenta cerca de 75 espécies de hábito herbáceo e arbustivo com centro de diversidade no Brasil e África subsaariana (Mohlenbrock 1961, Cardoso et al. 2013, Fortuna-Perez et al. 2013).

Estudos focados na filogenia do Clado Adesmia, mostraram que *Zornia* é grupo irmão de *Poiretia* e *Amicia* (Lavin et al. 2001, Fortuna-Perez et al. 2013), enquanto *Adesmia* é monofilético e *Nissolia* aparece como parafilético juntamente a *Chaetocalyx* (Wojciechowski et al. 2004, Lavin et al. 2001). Com relação a presença de extensões de bainha do feixe no Clado Adesmia, Mendes et al. (2019) relataram a presença de folhas homobáricas e folhas heterobáricas para as espécies de *Poiretia.* No entanto, para os demais gêneros desse grupo não há informações na literatura.

Objetivos

Este trabalho teve como objetivo caracterizar comparativamente a ultraestrutura das células da bainha do feixe em folhas homobáricas e heterobáricas e investigar a influência da luz e disponibilidade hídrica no desenvolvimento e funcionamento das EBF, além de elucidar como se deu a evolução dessa característica anatômica dentro de um Clado de Leguminosae.

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CAPÍTULO I

SUBCELLULAR PECULIARITIES OF BUNDLE SHEATH CELLS IN HOMOBARIC AND HETEROBARIC LEAVES FROM LEGUME SPECIES

Fernanda Helena Palermo, Tatiane Maria Rodrigues

Abstract

In leaves of many Angiosperms, the bundle sheath (BS) extends towards the epidermis of both leaf surfaces dividing the mesophyll into isolated compartments. Leaves bearing bundle sheath extensions (BSE) are referred as heterobaric, while leaves lacking BSE are termed homobaric. In this work, we investigated the subcellular aspects of BS cells in homobaric and heterobaric leaves from legume species, using two arboreal species occurring in the Brazilian Cerrado as models: *Machaerium acutifolium* Vogel with heterobaric leaves and *Dimorphandra mollis* Benth. with homobaric leaves. Leaflet blade samples were processed according to usual techniques in transmission electron microscopy. In *M. acutifolium*, the plastids in BSE cells were scanty, small, and contained poorly developed thylakoids. In *D. mollis*, the cloroplasts were larger and with well-structured grain, similarly to those found in the palisade and spongy parenchyma. The low concentration of cloroplasts in the BSE of heterobaric leaves seems to be related to the translucent appearance of these cells that facilitates the entry of light to the leaf mesophyll. Primary pit fields with plasmodesmata connected the BSE cells between themselves and to the adjacent tissues, including the epidermis in *M. acutifolium*, reinforcing the idea of facilitating the lateral transport of water from the veins in heterobaric leaves. However, the primary pit fields were more abundant, and plasmodesmata were larger in *D. mollis*. So, the symplastic route for transport of water and other substances between veins and mesophyll seems to be more favored in *D. mollis*. The observation of intercellular spaces between BSE and chlorophyll parenchyma cells in *M. acutifolium* suggests that water and other substances can diffuse freely in the mesophyll via the apoplastic route in heterobaric leafed species. Considering that our data refer to a restricted number of plant species, research involving additional species is necessary for expanding our findings in a generalized way to species with homobaric and heterobaric leaves.

Key-words: bundle sheath, bundle sheath extensions, plasmodesmata, cloroplasts, ultrastructure.

Introduction

In leaves of several plant species, parenchyma and/or sclerenchyma cells that surrounds the vascular bundles project towards the epidermis (Esau 1977) forming bundle sheath extensions (BSE). Leaves bearing BSE around the veins projecting to both sides of the leaf blade are named heterobaric (Wylie 1952, Liakoura et al. 2009). In these leaves, the mesophyll is divided into small compartments called areolas or BSE compartments (Terashima 1992; Liakoura et al. 2009). In paradermal sections, these compartments are polygonal and establish a mesh along the entire length of the leaf blade. On the other hand, leaves devoid of BSE are called homobaric (Terashima 1992) and, in this case, the leaf mesophyll is not compartmentalized.

The cells that compound the BSE are juxtaposed (Wylie 1952; Terashima 1992), and display important physiological, biochemical and structural roles in the leaves (Kenzo et al. 2007, Terashima 1992, Bukley et al. 2011). According to Karabourniotis et al. (2000), BSE cells are usually translucent and act in the conduction of photosynthetically active radiation to more internal portions of the leaf mesophyll. In addition, BSE can affect water distribution in heterobaric leaves by reducing the hydraulic resistance between vascular bundles and the epidermis, including the stomata (Buckley et al. 2011). According to Terashima (1992), the compartmentalization of the mesophyll by BSE causes heterogeneity in the rates of gas exchange in heterobaric leaves, with the stomata subject to the control exercised by each compartment. Thus, each compartment can respond differently to environmental conditions.

Comparative information is lacking concerning the ultrastructural aspects of the bundle sheath (BS) cells in homobaric and heterobaric leaves. In a broader sense, it has been assumed that leaves with differential anatomical features and photosynthetic metabolisms exhibit bundle sheath cells with ultrastructural peculiarities. Studies have reported the presence of chloroplasts with well-structured grana and bulky starch grains in bundle sheath cells of C4 plants in contrast to C3 plants that admittedly do not have plastids with developed thylakoids (Robinson-Berrs & Evert 1991, Taniguchi et al. 2003, Omoto et al. 2009, Rogowski et al. 2019). The increased development of symplastic communication structures between bundle sheath and the mesophyll cells in C4 plants in comparison to C3 plants is another aspect that has been reported in the literature (Botha 1992, Ajao et al. 2017). Considering that species bearing homobaric and heterobaric leaves display different photosynthetic behavior (Nikolopoulus et al. 2002, Pieruschka et al. 2005, Kenzo et al. 2007, Inoue et al. 2015, Rodrigues et al. 2017), we hypothesized that the bundle sheath cells could exhibit distinctive ultrastructural aspects in these plant groups. So, our aim was to investigate the subcellular characteristics of bundle sheath cells in homobaric and heterobaric leaves using legume species as models.

Material and Methods

Plant material

In this study, we used two tree species from the Brazilian cerrado. *Dimorphandra mollis* Benth. presented bipinnately compound leaves with seven to nine leaflets (Ferreira et al 2001) with anatomical features corresponding to homobaric leaves. *Machaerium acutifolium* Vogel presented compound leaves with nine to 15 leaflets (Polido & Sartori 2007) bearing anatomical features corresponding to heterobaric leaves.

We collected fully expanded leaves located at the third node of peripheral stem branches of the crown from three individuals of *D. mollis* (n = 3) and *M. acutifolium* (n = 3) growing on a remnant of Cerrado (22°48'35" S, 48°39'57" W) in Pratânia municipality, São Paulo State, Brazil. This area has subtropical climate with dry winters, and mean temperature higher than 18ºC.

Vouchers were deposited in the herbarium Irina Delanova de Gemtchujnicov (BOTU) of the Institute of Biosciences of Botucatu from São Paulo State University (UNESP).

Light microscopy

Samples from the middle region of the leaflet blade were fixed in a mixture of formalin/acetic acid/ethanol 50% for 48 h (Johansen 1940), dehydrated in ethanol series, and embedded in methacrylate resin (Gerrits 1991). Serial cross sections (5 µm thickness) were obtained using a rotary microtome and stained with Toluidine blue pH 4.7 (O'Brien et al. 1964). Permanent slides were mounted with Entellan® and analyzed under a DMR photomicroscope (Leica, Germany) coupled with a digital camera.

Transmission Electron Microscopy (TEM)

Portions from the middle region of the leaflet blade were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 24 h, post-fixed with 1% osmium tetroxide in the same buffer for 1 h, dehydrated through acetone series, and embedded in Araldite resin (Machado and Rodrigues 2004). Ultra-thin sections were post-stained with uranyl acetate and lead citrate (Reynolds 1963). The material was examined using a Fei Tecnai transmission electron microscope at 80 kV.

Results

The homobaric leaflets of *Dimorphandra mollis* (Fig. 1a, b) were hypostomatous with uniseriate epidermis. The epidermal cells on the adaxial surface of the leaflet blade were voluminous with mucilaginous secretion. The mesophyll was dorsiventral and presented one layer of palisade parenchyma and two to three layers of spongy parenchyma (Fig. 1a, b). The veins immersed in the mesophyll were collateral vascular bundles surrounded by a sheath of parenchyma cells (Fig. 1a, b).

Ultrastructurally, the parenchyma cells of the bundle sheath of *D. mollis* were voluminous, juxtaposed, and irregular in shape (Figs 1c, d). The lateral ends of these cells exhibited intrusive growth (Fig. 1c, d). The walls of BS cells were thin, and the middle lamellae was thick and filled the intercellular spaces between themselves and between BS cells and the vascular bundle (Fig. 1c, d). Abundant and large primary pit fields with wide plasmodesmata were observed connecting the BS cells to vascular parenchyma (Fig. 1e) BS cells to BS cells (Fig. 1f, g) and BS cells to chlorophyll parenchyma cells (Fig. 1e).

The vacuoles of BS cells were well-developed and occupied most of the cell volume (Fig. 1c-f). Flocculant material, membranous debris and large dark bodies occurred inside the vacuoles (Fig. 1d-f). Small mitochondria (Fig. 1d), endoplasmic reticulum and cloroplasts occurred in the peripheral cytoplasm (Fig. 1d). The cloroplasts were lenticular and exhibited structured grana (Fig. 1d-g); starch grains and oil drops occurred inside the cloroplasts (Fig. 1d, e).

The cloroplasts in the BS cells exhibited similar morphology and size to those occurring in the neighboring parenchyma cells of the mesophyll (Fig. 1c, f, h). The palisade and the spongy parenchyma cells (Fig. 1h, i) had thin walls and dense cytoplasm. The vacuoles contained flocculant material and large dark bodies (Fig. 1h). Mitochondria and cloroplasts occupied the peripheral cytoplasm (Fig. 1h, i). The cloroplasts were abundant, large and irregular in contour and contained well-structured grana. Large starch grains, oil drops and dark plastoglobules occurred inside the cloroplasts (Fig. 1h, i).

The heterobaric leaflets of *Machaerium acutifolium* presented uniseriate epidermis with stomata on the abaxial surface (Fig. 2a, b). The mesophyll was dorsiventral with a layer of palisade parenchyma and three to four layers of spongy parenchyma (Fig. 2a). Collateral vascular bundles occurred immersed in the mesophyll (Fig. 2a). The bundle sheath surrounding the second, third and fourth order veins (Fig. 2a, b) projected towards the epidermis of both sides of the leaf blade, segmenting the mesophyll into compartments. Such BSE were compound of parenchyma and sclerenchyma cells (Fig. 2a, b). Fiber caps occurred associated with second and third order vascular bundles (Fig. 2a, b) and isolated lignified cells occurred in the minor veins.

The bundle sheath cells of *M. acutifolium* were round to oval in shape (Fig. 2c, d), juxtaposed and exhibited thick pectin-cellulosic cell walls (Fig. 2d, g). Cells with thick and lignified walls occurred intermixed with the cells with pectin-cellulosic walls were found in the bundle sheath (Fig. 2c, d). We observed primary pit fields with plasmodesmata between the parenchyma BSE cells (Fig. 2e), and between the BSE cells and the epidermal cells (Fig. 2f). However, we could not able to observe plasmodesmata connecting the BSE cells and the chlorophyll parenchyma cells. The BSE parenchyma cells exhibited reduced cytoplasm and voluminous vacuoles with electron-dense inclusions (Fig. 2e-h). Their plastids were scanty, small and had relatively few thylakoids (Fig. 2f, g); small starch grains and abundant plastoglobules occurred in the cloroplasts (Fig. 2g, insert). Both the palisade and spongy parenchyma cells had many cloroplasts in the peripheral cytoplasm (Fig. 2h, i). These cloroplasts were voluminous, lenticular in shape and contained well-structured thylakoids, starch grains and small plastoglobuli (Fig. 2i).

Discussion

Our data points to differential subcellular aspects when comparing the bundle sheath cells in species belonging to the same plant group (Angiosperm, eudicots, Leguminosae), with the same photosynthetic metabolism (C3), but bearing heterobaric and homobaric leaves. In the homobaric leafed *D. mollis*, we observed that the BS cells showed characteristics more similar to spongy and palisade parenchyma cells of the leaf mesophyll, mainly concerning the cloroplasts that were more voluminous, abundant and with structured grain. In addition, the BS cells of *D. mollis* exhibited very conspicuous plasmodesmata connecting themselves and with the neighboring tissues. In the heterobaric leafed *M. acutifolium*, the BSE cells presented scarcer plastids; when present, they were smaller and with poorly structured thylakoids. The plasmodesmata in these cells were fewer and smaller than in *D. mollis*.

In *D. mollis* the cloroplasts of the BS cells have similar size and morphology to those occurring in the neighboring chlorophyll parenchyma cells of the mesophyll. So, the BS of *D. mollis* is similar to that classified as L-type bundle sheath cells, where the chloroplast volume and number per unit volume are similar to those of mesophyll cells (Williams et al. 1989; Leegood 2008). On the other hand, *M. acutifolium* presented smaller chloroplasts than the mesophyll parenchyma cells. The BS cells containing chloroplasts with approximately a third of the volume of mesophyll chloroplasts are termed S-type Williams et al. 1989; Leegood

2008). Williams et al. (1989) proposed a differential role for barley L-type and S-type bundle sheath cells, suggesting that S-type cells are more effectively involved in the transport of assimilates to the phloem. If there is a differential involvement of the BS cells in the transport of assimilates in homobaric and heterobaric leaves experimental studies are necessary to confirm this hypothesis.

The presence of chloroplast in BS cells is a common feature among C3 plants (Leegood 2008). The fact is that both the species here studied have chloroplasts in the sheath cells; however, in *D mollis* the chloroplasts are larger and more abundant. The presence of chloroplasts in the BS cells suggests that in addition to their conductive function, these cells can have a collaborative role to the photosynthetic apparatus of the plant (Haberlandt 1914, apud Leegood 2008). However, studies have demonstrated that the concentration of chloroplasts in the BS cells seems to be significantly larger in C4 plants (see Leegood 2008). Based on our ultrastructural analyses, we suggest that the BS cells of homobaric leaves of C3 plants have structural and functional features more similar to those described for C4 plants than the heterobaric leaves of C3 plants.

The low concentration of cloroplasts in the BSE of heterobaric leaves seems to be related to the translucent appearance of these cells when observed under light microscopy (Mc Clendon 1992, Karabourniotis et al. 2000, Nikolopoulos et al. 2002, Liakoura et al. 2009). The cells that made up the BSE could perform another function that would be related to the entry of light to the leaf mesophyll (Liakoura et al. 2009) instead of actively participating in the photosynthesis process. Thus, the translucent aspect of these cells would facilitate the conduction of light to the palisade and spongy parenchyma cells (Liakoura et al. 2009), which have a greater number of chloroplasts with developed thylakoids, as we also observed in the species studied.

In both the homobaric leafed *D. mollis* and the heterobaric leafed *M. acutifolium*, the bundle sheath was comprised by a layer of compactly arranged cells surrounding the veins. The bundle sheath is considered a conduit between the vasculature and the mesophyll cells, conducting water and photosynthates into and out the leaves during different developmental stages, being the only cells outside the veins through which these substances must pass (Leegood 2008). In addition, in C3 plants, the structural features and juxtaposition of the bundle sheath cells are considered to maintain the hydraulic integrity of the leaves to prevent air from entering the xylem in the veins (Sage 2001). This feature is still more evident in the homobaric leafed *D. mollis* where the BS cells present ends fitted in an arrangement provided by intrusive growth.

The BSE cells of *M. acutifolium* were juxtaposed with each other and had a thicker cell walls, some with lignified walls. Plasmodesmata were fewer and smaller in this species.

Thickening and fouling of cell walls with hydrophobic compounds can act in waterproofing and also against the passage of CO_2 (Brown 1975, Mertz & Brutnell 2014).

The presence of juxtaposed cells forming vertical lines in the leaf blade divides the mesophyll of heterobaric leaves in small and isolated compartments (Rodrigues et al. 2017, Boeger et al 2016, Mendes et al. 2016) and thus seem to restrict the lateral movement of gases through the leaf blade (Terashima 1992; Pieruschka *et al.* 2005). The formation of these small compartments in the leaf mesophyll would concentrate gases such as CO_2 and could increase carbon assimilation rates in species with heterobaric leaves, thus conferring an adaptive advantage to these species (Terashima 1992, Liakoura et al 2009). So, the observation of thick-walled and juxtaposed cells with poorly developed plasmodesmata in *M. acutifolium* can corroborate the idea of isolation of the compartments in the mesophyll of heterobaric leaves, restricting the lateral circulation of gases and becoming the gas exchange rates independent in each mesophyll compartment (Pieruschka et al. 2006; Buckley et al. 2011). In addition, the BSE are considered to reduce the spread of disease inside the leaves (Leegood 2008).

In the plant body, water can move through in plant tissues by apoplastic route (through the intercellular spaces and cell walls), symplastic route (via plasmodesmata), and transmembrane route (crossing plasmalemma) and the structural peculiarities of the cells and tissues are directly related to these pathways (Sevilem et al. 2013). Peculiarities on the hydric conductance have been reported between species with homobaric and heterobaric leaves and the involvement of BSE cells in the water conduction though the leaf blade has been reported for heterobaric leaves (Scoffoni et al. 2008, Buckley et al. 2011, Sommerville et al. 2012, Zsögon et al. 2015, Barbosa et al. 2019). In the heterobaric leafed *M. acutifolium*, we observed that plasmodesmata were smaller and fewer between BSE cells and between BSE and neighboring cells when compared to the homobaric leafed *D. Mollis*, suggesting a lower symplastic communication between the veins and the mesophyll in heterobaric leafed species. However, we observed intercellular spaces between BSE and chlorophyll parenchyma cells suggesting that water and other substances can diffuse freely in the mesophyll via the apoplastic route in heterobaric leafed species. Higher values for water use efficiency (*WUE*) in species with heterobaric leaves when compared to homobaric ones were observed in studies with temperate species (Liakoura et al 2009, Inoue et al. 2015) and are related to low transpiration rates and high carbon assimilation by the plants (Lynch et al. 2012).

Studies suggest that BSE could facilitate the water movement in the leaf blade from the vascular tissues until the epidermis (Buckley et al. 2011), decreasing the hydraulic resistance in the leaf blade (Buckley *et al.* 2011). In fact, the extension of the BS toward the epidermis and the presence of plasmodesmata connecting the BSE and epidermal cells in *M. acutifolium* reinforces the idea of facilitating water transport from the veins. The lateral water movement in heterobaric leaves, from the vascular system, passing through the BSE cells until reaching the epidermis cells would reduce the chances of dehydration in leaf tissues, especially in conditions of high solar radiation or low water availability (Buckley et al. 2011, Barbosa et al. 2019), representing a more efficient pathway to water flux in the mesophyll (Wylie 1952).

On the other hand, we observed that the symplastic route among veins-BS-mesophyll cells seems to be more favored due to the abundance of wider and abundant plasmodesmata connecting BS cells between themselves and to the neighboring tissues in the homobaric leafed *D. mollis*. Plasmodesmata are known to form a low-resistance pathway between two cells through which large hydrophilic molecules can travel faster (Wayne 2010), facilitating the exchange of water and metabolites via the symplast (Danilla et al. 2018), what could favor the lateral communication between cells in the homobaric leaves. In addition, in *D. mollis*, the juxtaposition of the BS cells, their intrusive growth and the thick middle lamella could hinder the apoplastic route. Although there seems to be a more efficient symplastic transport in homobaric leaves, the lack of compartmentalization of the mesophyll and the spatial discontinuity between the veins and the epidermis could be related to the lower efficient water use and higher transpiration rates in homobaric leaves (Scoffoni et al. 2008) and would allow greater lateral diffusion of gases in the leaf mesophyll (Kenzo *et al.* 2007, Liakoura *et al.* 2009).

Our ultrastructural analysis highlighted peculiarities on the subcellular features of the BSE cells in heterobaric leaves and BS cells in homobaric leaves and evidenced the relation of such structural features with the physiological properties of the leaves. However, our data are still preliminary and based on a very restricted number of plant species. Further data considering additional species will be necessary for expanding our findings in a generalized way to species with homobaric and heterobaric leaves.

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Figures and legends

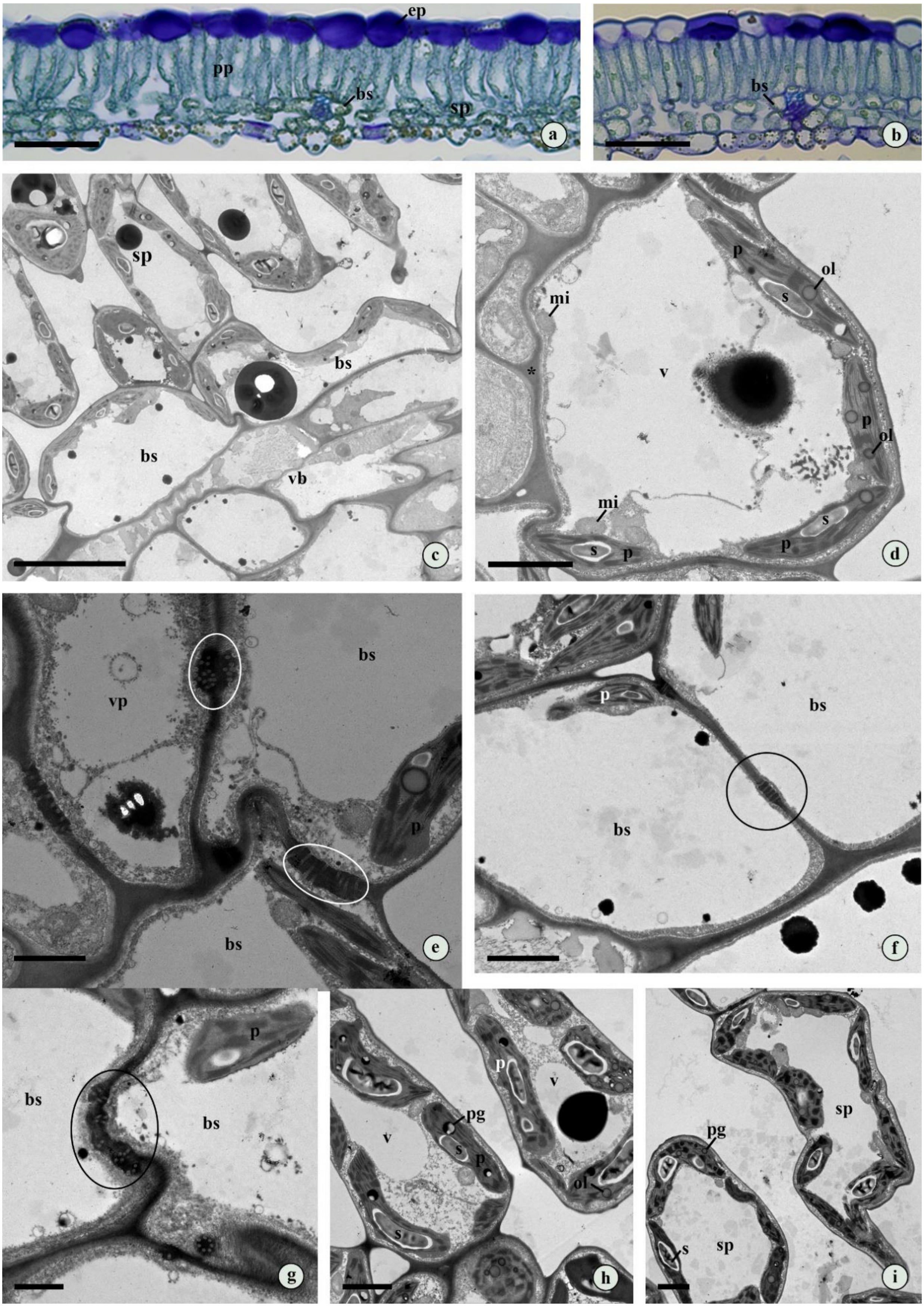

Fig. 1. Light **(a, b)** and transmission electron **(c-i)** micrographies of *Dimorphandra mollis* leaflet blade. **a.** Homobaric leaf with uniseriate epidermis (ep), mesophyll with palisade (pp) and spongy (sp) parenchyma and vascular bundle surrounded by bundle sheath (bs) without cell extensions. **b.** Detail of vascular bundle surrounded by bundle sheath (bs) parenchyma cells. **c.** Parenchyma bundle sheath (bs) cells with intrusive growth. sp: spongy parenchyma; vb: vascular bundle. **d.** Detail of bundle sheath cell with large vacuole (v) with dark inclusions, and reduced cytoplasm containing mitochondria (mi) and cloroplasts (p) with well-developed thylakoids, starch grains (s) and oil droplets (ol). Observe the swollen middle lamellae (*) filling the regions between BS cells and their neighbors. **e.** Primary pit fields (encircled) with wide plasmodesmata connecting the bundle sheath cells (bs) between themselves and to the vascular parenchyma (vp). p: plastid. **f.** Primary pit fields (encircled) with wide plasmodesmata connecting the bundle sheath cells (bs) between themselves. g. Observe the larges plasmodesmata (encircled) connecting bundle sheath (bs) cells. p: plastid. **h-i.** cloroplasts (p) with thylakoids, starch grains (s) and plastoglobules (pg) in palisade parenchyma cells (h) and spongy parenchyma cells (i). Scale bars: 100 µm (a, b); 10 µm (c); 5 µm (d, f, h, i); 2 µm (e, g)

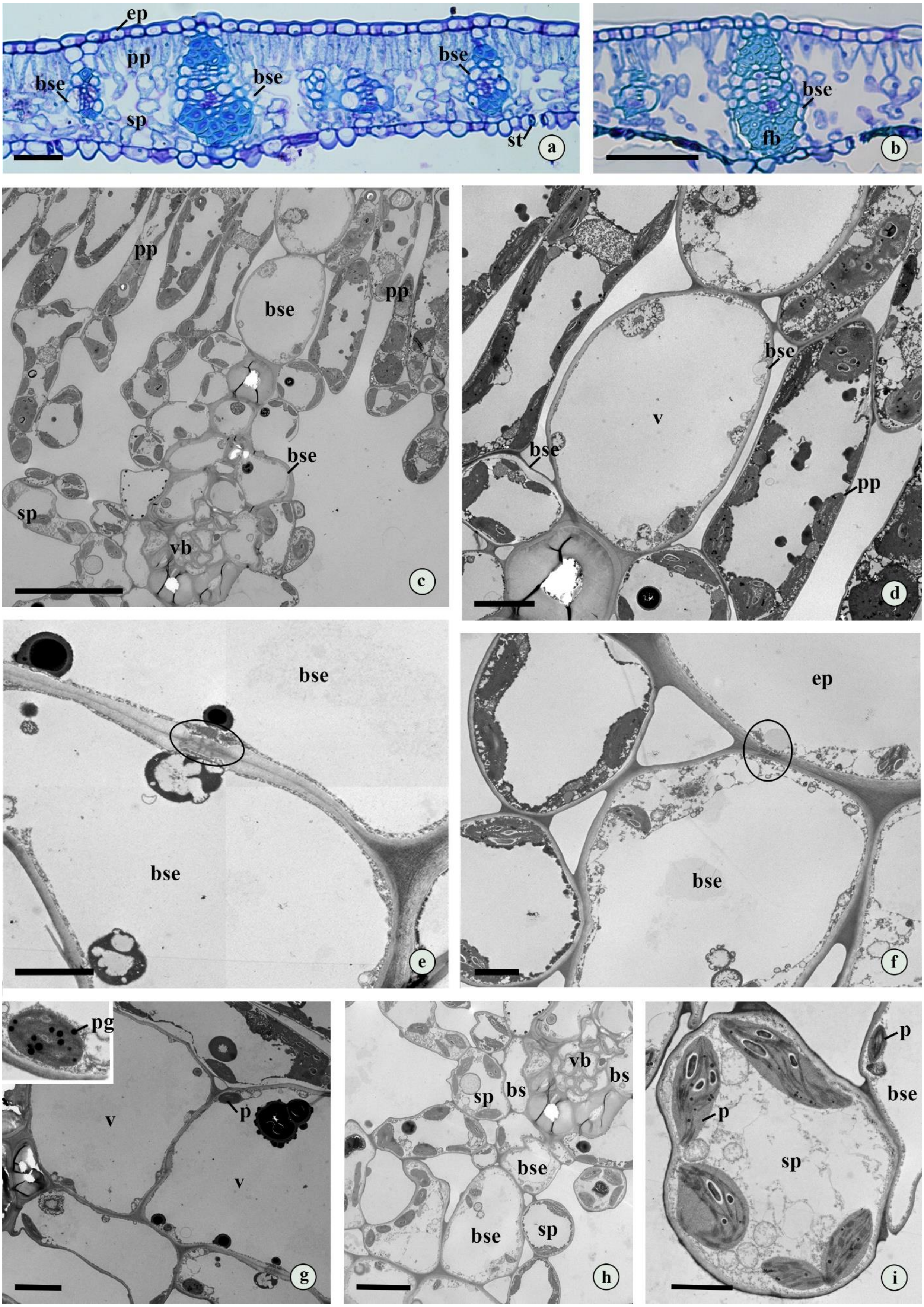
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Fig. 2. Light **(a, b)** and transmission electron **(c-i)** micrographies of *Machaerium acutifolium* leaflet blade. **a.** Heterobaric leaf with uniseriate epidermis (ep), mesophyll with palisade (pp) and spongy (sp) parenchyma and vascular bundles surrounded by bundle sheath extending toward the epidermis. bse: bundle sheath extension; st: stomata. **b.** Detail showing vascular bundle sheath extension (bse). fb: fibers. **c.** Leaflet portion with palisade (pp) and spongy (sp) parenchyma and vascular bundle (vb). bse: bundle sheath extension. **d.** Bundle sheath extensions (cells) crossing the mesophyll. pp: palisade parenchyma. V: vacuole. **e.** Bundle sheath extension cells (bse) connected by primary pit field (encircled). **f.** Primary pit field (encircled) between bundle sheath extension cell (bs) and epidermis (ep).**g.** BSE cells with large vacuoles (v) containing electron-dense inclusions and small cloroplasts (p). In the insert, a plastid with poorly developed thylakoids and plastoglobuli (pg). **h.** Overview showing spongy parenchyma cells (sp) with large cloroplasts and bundle sheath extensions cells (bse) with few and small cloroplasts. **i.** Detail of spongy parenchyma cell with large cloroplasts (p) in peripheral cytoplasm and portion of bundle sheath extension cell (bse) with small plastid (p). Scale bars: 100 µm (a,b); 50 µm (c); 20 µm (h); 10 µm (d, g); 5 µm (e, f, i)

CAPÍTULO II

MORPHOPHYSIOLOGICAL ASPECTS OF HOMOBARIC AND HETEROBARIC LEAVES OF LEGUMES FROM THE BRAZILIAN SAVANNA UNDER DISTINCT LIGHT AND WATER AVAILABILITIES

Fernanda Helena Palermo, Davi Rodrigo Rossatto, Felipe Girotto Campos, Tatiane Maria Rodrigues

Abstract

Vascular bundles immersed in the leaf mesophyll are surrounded by juxtaposed cells forming a sheath. In several plant species, this sheath can be projected to both sides of the leaf blade, constituting bundle sheath extensions (BSE). Leaves with BSE are classified as heterobaric leaves, while those lacking BSE are called homobaric. The presence/absence of BSE in the leaf blade is a fixed character; however, the distance between BSE can change according to environmental conditions. Studies involving species from temperate climates have shown a reduction in BSE spacing in heterobaric leaves subjected to higher light intensity and/or lower precipitation, suggesting a more efficient water use and higher carbon assimilation. However, there is a lack of knowledge for tropical plants showing these structures. In this study, we aimed to investigate the influence of light and water availability on the distancing between veins and on gas exchange rates in homobaric and heterobaric leaves in two legume species from the Brazilian savanna. Individuals of the heterobaric leafed *Machaerium acutifolium* Vogel and of the homobaric leafed *Dimorphandra mollis* Benth. were submitted to shade/full sun treatments and also to water availability at the field capacity and water restriction in greenhouse experiments. Leaflet blades were cross sectioned and the distance between veins was measured under light microscopy. The gas exchanges values, leaf water potential and light response curves were registered. For both species, the distance between vascular bundles did not change among the treatments, what seems related to their adaptation to the environmental conditions of the Brazilian savanna. On the other hand, *M. acutifolium* showed lower rates of assimilation, transpiration, and stomatal conductance in comparison to *D. mollis*, regardless of the treatment to which they were submitted. In addition, *M. acutifolium* showed higher values of water use efficiency both under field capacity and water restriction. Our results showed that physiological results can be associated with inherent strategies of heterobaric and homobaric leafed species for the use of water and light.

Keywords: bundle sheath extensions, gas exchange, veins, water use efficiency

Introduction

Leaves are the main photosynthetic organ of vascular plants and exhibit anatomical characters related to this important physiological process (Brazel et al. 2019). Specializations related to gas exchange, light absorption, reduction of water loss and transport of substances are commonly observed in leaf tissues, and their variations in size or length are likely associated with environmental conditions (Dickison 2000). In fact, most of leaf structures can be very plastic concerning variations in temperature, water availability and light (Gratani 2004).

In most vascular plants, leaves have collateral bundles that branch into veins of different sizes (Esau 1960). Such veins are surrounded by a sheath of specialized cells, named endodermis (Menezes 1971; Menezes et al. 2001) or vascular bundle sheath (Dickison 2000). Such structure is physiologically important for water and photoassimilates exchanges between vascular tissues and the leaf mesophyll cells (Dickison 2000). In several plant species, parenchyma or sclerenchyma cells from the sheath surrounding the vascular bundles can be projected towards the epidermis constituting bundle sheath extensions (BSE). Leaves showing BSE projecting to both sides of the leaf blade are named heterobaric (Wylie 1952, Liakoura et al. 2009) and have the mesophyll divided into small compartments (Terashima 1992, Inoue et al. 2015). In this case, the cells that comprise the BSE are juxtaposed and are usually free of photosynthetic pigments (Wylie 1952; Terashima 1992, Zsögon et al. 2015). On the other hand, leaves without BSE are called homobaric and have a homogenous mesophyll without compartmentalization (Terashima 1992).

Photosynthetic rates of each compartment in heterobaric leaves seem to be higher when compared to homobaric leaves (Nikolopoulos et al. 2002). Species with heterobaric leaves, when exposed to conditions of high irradiance (Scoffoni et al. 2008) or low humidity (Buckley et al. 2011) seems to present a lower hydraulic resistance between the vascular bundles and the epidermis, which is coupled with lower stomatal conductance (Zsögon et al. 2015). Thus, it is expected that under stress conditions, species with heterobaric leaves would have better water balance since the BSE would facilitate the water transport through the leaf blade parenchyma (Buckley et al. 2011, Barbosa et al. 2019).

The presence/absence of BSE in the leaf blade is a fixed character and the size of the compartments formed in the mesophyll of heterobaric leaves is related to the order of leaf vein showing the BSE, which can vary according to plant species (Rodrigues et al. 2017). However, considering that the density of leaf veins is a plastic feature, the spacing of BSE in the mesophyll of heterobaric leaves can vary according to environmental conditions such as light and water availability (Lynch et al. 2012). Few studies dealing with the theme have shown a reduction in

BSE spacing in heterobaric leaves of temperate tree species subject to higher light intensity (Wylie 1951; Lynch et al. 2012) and lower precipitation (Lynch et al. 2012). Such changes suggest that these leaves can show better water use efficiency, since under stress conditions these plants can maintain their carbon assimilation rates, which was also related to the higher amount of ^{13}C in most of the species studied (Lynch et al. 2012).

Therefore, we argued if plastic adjustments on the BSE spacing in response to environmental conditions could also occur in leaves of species from tropical systems, such as the Brazilian savannas (The Cerrado) where many plants have BSE (Rodrigues et al. 2017); and if these changes could drive variations in leaf photosynthetic capacity. Unlike from the temperate forest species, Brazilian savanna species naturally cope with acid soils deficient in nitrogen and phosphorus and with high levels of aluminum (Simon and Pennington 2012). The Brazilian savanna vegetation occurs in areas with significant dry season where fire is an important ecological factor (Pennington et al. 2006). Most of the trees from the Brazilian savanna are generally evergreen and their root systems are profound in the ground (Franco 2002, 2005). This depth root system coupled with relatively small leaves and greater stomatal control of transpiration allows Brazilian savanna trees to maintain positive water balance throughout the year (Franco 2005).

In this paper, we aimed to investigate the influence of light and water availability (under an experimental approach) on the distancing between the minor veins and to evaluate comparatively gas exchange rates in homobaric and heterobaric leaves in phylogenetically related legume species which occur side by side in seasonal environments of the Brazilian savanna. We hypothesized that (1) woody heterobaric leafed species would exhibit smaller spacing between the BSE in response to light and water availability and (2) heterobaric leafed species will present higher carbon assimilation rates, lower stomatal conductance values and greater efficiency of water use under higher light intensity and lower water availability in comparison with homobaric leaves.

Material and methods

Plant material

Two tree species of Leguminosae with C3 photosynthetic metabolism, commonly found in the Brazilian savanna (Sano et al. 2008, Flora do Brasil 2020) were used as a model for this study: *Machaerium acutifolium* Vogel (Faboidea), having heterobaric leaves and *Dimorphandra mollis* Benth (Mimosoidea) showing homobaric leaves. *M. acutifolium* is characterized by a strictly arboreal habit and presents nine to 15 foliolate leaves (Polido & Sartori 2007). *D. mollis* has bipinnately compound leaves with seven to eight leaflets (Ferreira et al 2001).

Approximately one-year-old plants of *M. acutifolium* were obtained from a plant nursery in Assis municipality (22º 39' 42" S and 50º 24' 44" W), São Paulo State, Brazil. Young plants of *D. Mollis* were obtained from a plant nursery in Alto Paraíso de Goiás city (14° 07' 58" S, 47° 30' 36" W), Goiás State, Brazil. The plants were grown in pots with a capacity of 10L at 26ºC ± 4ºC in a greenhouse located in the Institute of Biosciences of Botucatu (IBB), São Paulo State University (UNESP), Botucatu municipality (22º 53’ 09 S, 48° 26’ 42” W), São Paulo State, Brazil, located at about 786m of altitude. The experiment was conducted between June and November of 2020. This region has a warm and humid temperate climate (Cunha et al. 2009) with average temperatures around 20.3ºC, reaching up to 27.3ºC during the rainy season, which corresponds from October to March. The average annual rainfall is around 1501 mm (Cunha et al. 2009).

After an acclimatization period of two months, the plants were submitted to different conditions of light and water availability. To analyze the influence of luminosity on homobaric and heterobaric leaves, ten plants of each species (n =10) were submitted to two treatments: a) 200 μ mol m^{-2} s^{-1}, which is equivalent to the average irradiance measured in the shaded understory of the Brazilian savanna (Pinheiro et al. 2016); and b) approximately 1000 μ mol m^{-2} s^{-1}; according to Prado & De Moraes (1997) this rate is sufficient to reach 90% of photosynthesis saturation in tree species.

To analyze the influence of water availability on homobaric and heterobaric leaves, plants from both species (n =10) were submitted to: a) soil field capacity (FC), under constant irrigation; and b) water restriction (WR) in which irrigation was suspended until the stomatal conductivity rates reached values referring to 30% in relation to the plants in the field capacity (FC) treatment.

Both experiments were performed simultaneously, and the experimental design consisted of 10 randomized blocks with two replications of each species, totalizing eight individuals per block (Fig. 1).

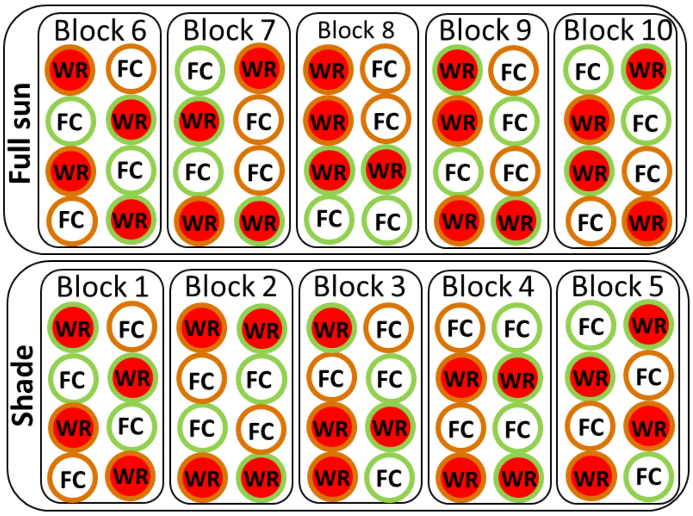

Fig 1. Experimental design. Green circles represent individuals of *Machaerium acutifolium* and orange circles represent individuals of *Dimorphandra mollis*. FC = water field capacity; WR = water restriction.

Gas Exchange Measurements

To evaluate the physiological performance of species with different leaf types under the experimental conditions, gas exchange values were measured using an infrared gas analyzer - IRGA (Gas Analyzer - IRGA, GFS-3000 WALZ). Measurements were performed on fully expanded leaves formed after the application of treatments from three individuals per species (n =3), on sunny days, from 9:00 am to 11:00 am (Campos et al. 2019). The gas exchange parameters collected were: CO_2 assimilation rate (*A*, µmol CO_2 m^{-2} s^{-1}), transpiration rate (*E*, mmol water vapor m^{-2} s^{-1}), stomatal conductance (*gs*, mmol m^{-2} s^{-1}) and water use efficiency (*WUE*, mmol H_2O). These variables were calculated by the data analysis program of the photosynthesis measuring equipment that uses the general gas exchange equation of Von Caemmerer & Farquhar (1981). The reference CO_2 concentration used during the evaluations was that present in the environment (421 ppm) and, to homogenize the repetitions, the photosynthetic photon flux density (PPFD) was generated by a light-emitting diode coupled to the photosynthesis chamber, standardizing the luminosity (1200 µmol m^{-2} s^{-1}) that was present in the environment in each evaluation period. Water use efficiency (*WUE*, µmol CO_2 (mmol H2O)$^{-1}$) was determined by the relationship between CO_2 assimilation rate and transpiration rate (*A/E*) as described by Zhang et al. (2001).

Light response curve

For light response curve measurements, data were collected using an infrared gas analyzer - IRGA (Gas Analyzer - IRGA, GFS-3000 WALZ). Fully expanded leaves without signs of senescence or damage formed after the application of the treatments in three individuals per species (n =3), were placed in the IRGA chamber (8 cm^2) at average environmental concentrations of CO_2 (420 ppm), relative humidity (≈50 %), and temperature (27°C). *Macherium acutifolium* and *D. mollis* leaflets were placed until the equipment chamber was filled, without juxtaposition. We used a 10-level photosynthetic photon flux density (PPFD) generated by a blue-red LED light source in the following order: 0, 50, 100, 200, 500, 800, 1100, 1400, 1700, and 2000 $\mu molm^{-2}s^{-1}$. The net photosynthetic rate (*A*) in each PPFD was stored once these values stabilized. Measurements were taken during the morning hours (9:00-11:30 h).

A non-rectangular hyperbola was fitted to the collected data (CO_2 based on assimilation area (*A*) as a function of PPFD) following Thornley (1976) and using the algorithms provided by Lobo et al. (2013). Based on the fitted curves, we extracted the values of *A*max – the maximum rate of saturating light per area [µmol (CO2) m^{-2} s^{-1}], the light compensation point - LCP (PPFD where *A*=0), and the light saturation point - LSP (Prioul & Chartier, 1977). The apparent quantum yield (AQY) was calculated as the initial slope of the light response curve. The convexity coefficient (CC – rate of curvature of the light response curve) was obtained from the non-rectangular hyperbola fitted to the data.

Leaf Water Potential (Ψw)

Fully expanded leaves formed after the installation of the experiments were collected from 5 individuals per species (n =5) in each treatment, totaling 40 samples. Then, the samples were fragmented and placed in the chamber (7 mL) of the Water Potential Analyzer with Temperature Controller, WP4-T (DECAGON DEVICES, USA) for readings referring to leaf water potential. The analyzes were performed in the morning from 9:00 am to 11:00 am.

Light microscopy

For the anatomical studies, fully expanded leaves originated after the installation of the experiments were collected from five individuals per species (n =5) in each treatment. Samples from the middle region of the leaflet blade were fixed in a mixture of formaldehyde, acetic acid and 50% ethanol (Johansen 1940), dehydrated in an ethanol series and embedded in methacrylate resin (Gerrits 1991). Cross sections (5 µm thick) were obtained on a RM2145 semiautomatic rotary microtome (Leica, Germany) and stained with 0.05% Toluidine blue pH 4.7 (O'Brien et al. 1964). Permanent slides were mounted with coverslips using synthetic resin and analyzed under a DMR light photomicroscope (Leica, Germany). The relevant results were documented using a digital camera coupled to the photomicroscope. The distance between the minor veins in the heterobaric and homobaric leaves was measured using the LAS software (Leica, Germany).

Data analysis

The morphometric data, gas exchange rates, leaf water potential and parameters extracted from the light curves were analyzed using the three-way ANOVA statistical test, comparing treatments and leaf types and the means compared using the Tukey test (5%). For the homogeneity of variances, Levene's test was used with the help of the SigmaPlot 12.0 statistical program.

Results

Anatomical features of leaflets and influence of environmental effects on the distance between veins

The leaflet blade of *M. acutifolium* presents uniseriate epidermis with stomata on the abaxial surface and dorsiventral mesophyll with a layer of palisade parenchyma and three to four layers of spongy parenchyma (Fig. 2a). Collateral vascular bundles occur immersed in the mesophyll and are surrounded by a bundle sheath constituted by juxtaposed parenchymatic and sclerenchymatic cells (Fig. 2a, b). In the second, third and fourth order veins, the bundle sheaths project towards the epidermis of both sides of the leaf blade, segmenting the mesophyll into compartments (Fig. 2a, b). Fiber caps occur associated with the second order vascular veins (Fig. 2a).

In *D. mollis*, the leaflet blade is covered by uniseriate epidermis containing stomata on the abaxial leaflet surface. On the adaxial surface of the leaflet blade, the epidermal cells are voluminous and contain mucilage. The mesophyll is dorsiventral with one layer of palisade parenchyma and two to three layers of spongy parenchyma (Fig. 2c, d). The small veins embedded in the mesophyll are collateral vascular bundles enclosed by a parenchyma bundle sheath devoid of extensions (Fig. 2d).

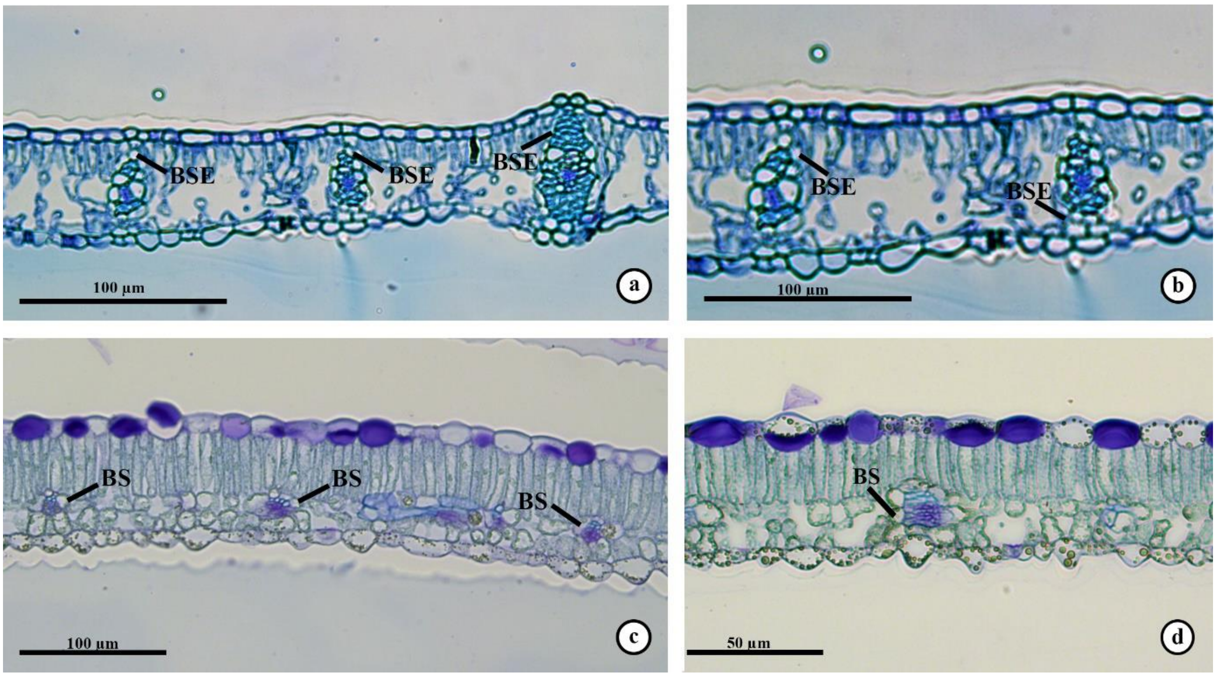

Fig. 2. Cross-sections of leaflet blades. **a, b** Heterobaric leafed *Machaerium acutifolium*. **c, d** Homobaric leafed *Dimorphandra mollis*. BSE = bundle sheath extension; BS = bundle sheath. Scale = 200µm.

The spacing between the vascular bundles immersed in the mesophyll was not affected by the experimental conditions of water availability and luminosity employed in this study for both species (F = 1,148, P = 0,328 and F = 1,283, P = 0,265, respectively) (Table 1), although the average distance between veins was higher in the heterobaric species in comparison with the homobaric one.

Table 1. Means ± standard error of distance between bundle sheath extensions (BSE) in individuals of *Machaerium acutifolium* and *Dimorphandra mollis* submitted to different conditions of water availability and luminosity according to factor analysis of variance.

Species	Distance between BSE (µm)
M. acutfolium (heterobaric)	192,3 ± 3,74
D. mollis (homobaric)	153,5 ± 4,29

Effect of light and water on gas exchange

Significant differences on CO_2 assimilation (A), transpiration (E) and stomatal conductance (gs) were registered between the species (F = 89.707, P <0.001; F = 68.320, P <

0.001; $F = 120.977$, $P = 0.001$, respectively for each parameter studied). However, treatment factors (water availability and luminosity) did not influence significantly these gas exchange parameters. *M. acutifolium* showed lower rates of assimilation (Fig. 3a), transpiration (Fig. 3b) and stomatal conductance (Fig. 3c) when compared to *D. mollis*, regardless of the treatment to which they were submitted. For water use efficiency (*WUE*), there was a significant interaction between the species and water factors ($F = 10.755$, $P = 0.005$), where *M. acutifolium* showed higher values both in field capacity (FC) and in water restriction (WR) (Fig. 3d).

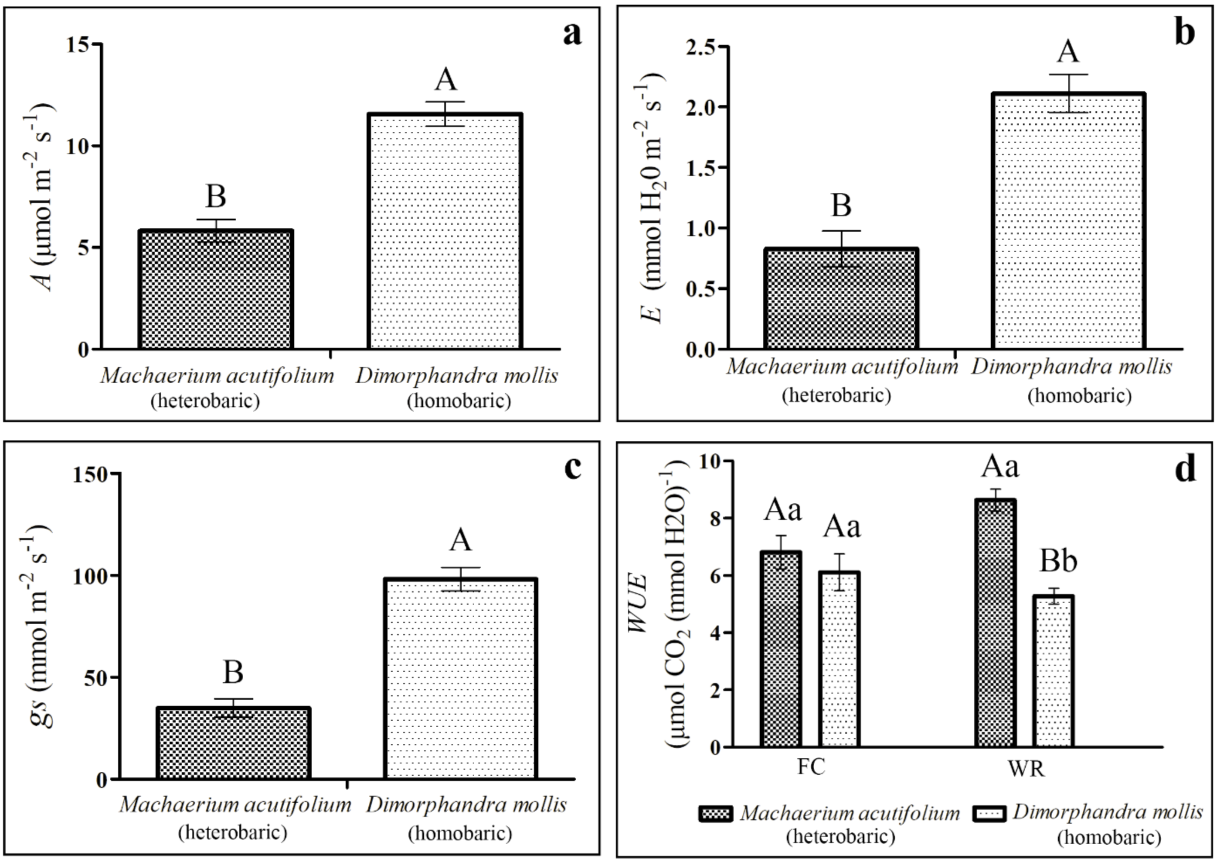

Fig. 3. Gas exchange data for *M. acutifolium* and *D. mollis*. Means ± standard error. **a.** CO_2 assimilation rate (*A*), **b.** Transpiration rate (*E*), **c.** Stomatal conductance (*gs*) and **d.** Water use efficiency (*WUE*). Three-way ANOVA followed by Tukey's test at 5%. In **d.**, capital letters indicate differences within the water factor. Lowercase letters indicate differences within the species factor (FC = field capacity, WR= water restriction).

Light response curves

Regarding the light response curves, the light compensation point (LCP) and the light saturation point (LSP) were similar for both species. However, *M. acutifolium* approached saturation at lower light levels than *D. mollis* (Fig. 4), although we found no statistical difference. The CO_2 assimilation rate was higher for *D. mollis* at different PPFD levels. The photosynthetic parameters extracted from the light response curves showed statistical differences for the apparent quantum yield (AQY) (F = 11.777 and P = 0.003) and for the maximum assimilation (*A*max) (F = 6.628 and P = 0.022). *Machaerium acutifolium* showed higher AQY; however, *D. mollis* showed higher *A*max values (Table 2).

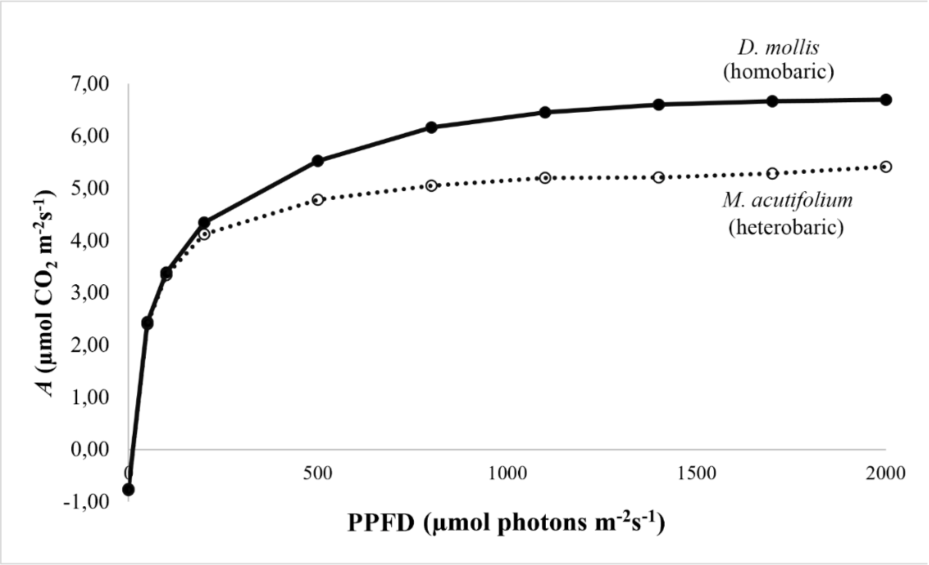

Fig. 4. CO_2 assimilation rate (*A*) vs. photosynthetic photon flux density (PPFD) in *Machaerium acutifolium* (heterobaric) and *Dimorphandra mollis* (homobaric).

Table 2. Means ± standard error (n=24) for the photosynthetic parameters analyzed for *M. acutifolium* (heterobaric) and *D. mollis* (homobaric) under different conditions of water and luminosity availability.

Parameters	*M. acutfolium* (heterobaric)	*D. mollis* (homobaric)
A_{max} µmol (CO2) m^{-2} s^{-1}	4,0467 ± 0,4893*	5,5267 ± 0,6931*
Rd µmol (CO2) m^{-2} s^{-1}	0,8081 ± 0,2233	0,5846 ± 0,1243
LCP µmol (photons) m^{-2} s^{-1}	6,2517 ± 1,6071	7,0075 ± 1,7626
LSP µmol (photons) m^{-2} s^{-1}	137,3 ± 28,77	196,3 ± 41,032
AQY µmol (CO2) μmol^{-1} (photons)	0,1468 ± 0,0141*	0,0953 ± 0,011*
CC	0,3172 ± 0,0839	0,34873 ± 0,0802

*A*max – Maximun assimilation rate; Rd – Dark respiration; LCP- Light compensation point; LSP – Light saturation point; AQY – Apparent quantum yield; CC – Convection coefficient.
* indicates statistical difference according to Tukey's test (p<0.001).

Leaf water potential (Ψw)

Regarding leaf water potential (Ψw) we found a significant interaction between the species and luminosity factors (*F* = 7.067, *P* = 0.012). *Machaerium acutifolium* showed higher values of Ψw when compared to *Dimorphandra mollis* (Table 3).

Table 3. Means ± standard error of leaf water potential (Ψw) between plants of *Machaerium acutifolium* and *Dimorphandra mollis* according to factorial analysis of variance.

Treatments	**Ψwf (Mpa)**	
	Machaerium acutifolium (heterobaric)	*Dimorphandra mollis* (homobaric)
Full sun	-2,193 ± 0,131 aA	-3,24 ± 0,07 aB
Shade	-2,096 ± 0,127 aA	-2,681 ± 0,101 bB

* Means followed by equal capital letters on the lines do not differ from each other. Means followed by lowercase letters in the columns do not differ by Tukey's test (5%). As there was no significant difference between the treatments in field capacity and water restriction, the two treatments were considered as one group.

Discussion

We observed that heterobaric and homobaric leafed species from the Brazilian savanna display different physiological behaviors in response to light and water conditions, although the distance between veins in the leaf mesophyll remains unchanged despite the water availability and luminosity variations.

Different light intensity conditions and water availability did not influence the distance between vascular bundles in both *M. acutifolium* and *D. mollis*. This data differs from those found on the literature for plants from temperate climate, where smaller compartments in the mesophyll with closer veins containing BSE occurred in plants submitted to high light intensity (Wylie 1951; Lynch et al. 2012) and lower water availability (Lynch et al. 2012). In environments with seasonal precipitation and soils with features that influence the water availability, as in the Brazilian savanna, the plants develop characteristics and mechanisms to deal with water scarcity. The maintenance of the distance between veins in the leaves may represent water use savings, since higher leaf vein density may incur significant water costs (Dios et al. 2021).

Concerning species of the Brazilian savanna, the presence of BSE in their leaves has already been reported as a common character in leaves of its species by several studies (Bieras & Sajo 2009, Rossatto et al. 2015, Ferreira et al. 2015, Mendes et al. 2016, Ariano & Silva 2016, Gonçales-Silva et al. 2019, Pessoa et al. 2019, Ariano et al. 2022). However, quantitative analyses on the BSE development were lacking, since the majority of studies involving ecological anatomy of Brazilian savanna plants measure features related to the size of structures such as the stomata, cuticle and mesophyll parenchyma cells (Rossatto et al. 2009, Fank-de-carvalho et al. 2010, Vieira Neto et al. 2020, Marinho et al. 2021). However, it is difficult to find studies that focus on the vascular density of the leaves, although the density of vascular bundles in the leaf blade seems related to higher light intensity and environmental conditions of xeric environments (Lopes et al. 2008), such as the Brazilian savanna.

In addition, it should be noted that we measured the distance between vascular bundles immersed in the mesophyll in cross sections of leaves, and that other studies have used frontal view images of leaves to measure the distance between the BSEs (Wylie 1951, Lynch et al. 2012). Analyzes of cross-sections with low thickness under the light microscope allow the visualization of the internal structure of the leaf blade in more detail and the unequivocal distinction between vascular bundles containing BSE and those lacking such structure, especially when dealing with smaller veins, which can lead to greater accuracy of the data collected.

The homobaric and heterobaric leafed species studied here, respond differently to variations in light conditions and water availability. Our results showed that heterobaric leafed *M. acutifolium* exhibits lower values of stomatal conductance and water use efficiency under higher light intensity and lower water availability compared to the homobaric leafed *D. mollis*, as suggested in our initial hypotheses. However, *D. mollis* showed higher values of carbon assimilation rate. Although both species are phylogenetically related belonging to Leguminosae and occur under the same environmental conditions in the Brazilian savanna (Mendonça et al. 1998, Flora do Brasil 2020), the presence/absence of BSE seems to allow different physiological responses to environmental variations.

Stomatal closure can be seen as a water saving strategy (Yang et al. 2021). This directly influences other values of gas exchange parameters, such as the transpiration and assimilation. Low values of stomatal conductance and transpiration may be related to the maintenance of water in the leaf, even when the plant is under stress situations, such as low water availability and high luminosity (Prado et al. 2004). The lower values of transpiration and stomatal conductance observed for *M. acutifolium* may indicate that this species has a more sensitive control of transpiration through stomatal closure, by reducing water loss to the atmosphere (Chaves et al. 2002). *Machaerium acutifolium* presented higher values of leaf water potential regardless of the treatment to which they were submitted, maintaining leaf turgor more efficiently even in situations of low water availability and high luminosity. Besides the stomata closure, the role of BSE in the maintenance leaf water potential has been pointed out in several studies (Liakoura et al. 2009, Buckley et al. 2011, Kawai et al. 2017, Rodrigues et al. 2017). The presence of BSE in heterobaric leaves can interfere with the hydraulic resistance between the vascular system and the epidermis, increasing the water flow in the leaf blade (Buckley et al. 2011, Scoffoni et al. 2008, Sommerville et al. 2012, Zsögon et al. 2014). So, heterobaric leaves would be more efficient in terms of water consumption, as water transport through the mesophyll would be facilitated by the presence of the BSE (Liakoura et al. 2009, Inoue et al. 2015).

The light response curves of both studied species showed that *M. acutifolium* tends to reach the LSP under conditions of lower light exposure than *D. mollis*, although we did not register a statistically significant difference for these values. The LSP indicates the moment when the plant stabilizes its light-dependent CO_2 assimilation process (Lobo et al. 2013). According to Karabourniotis et al. (2000), the BSE could act as “windows”, leading light into the leaf mesophyll, helping to capture light energy. Thus, the presence of BSE in heterobaric leaves could favor the transfer of light to the innermost layers of the mesophyll (Nikolopoulous et al. 2002), that could explain the behavior observed in the light response curves for the species in our study. In this sense, we also observed higher apparent quantum yield (AQY) values for heterobaric leaves, which would indicate greater efficiency in the use of light for CO_2 fixation (Rossatto et al. 2018) even when individuals are exposed to lower PPFD values. The reduction of the green area in the mesophyll due to the presence of translucent cells lacking chloroplasts in the BSE could represent a decrease of the photosynthetic area in heterobaric leaves. However, this condition would be equalized in heterobaric leaves by their improved absorption of light and water storage (Liakoura et al. 2009). Our results are consistent with this hypothesis, since *M. acutifolium* reached LSP under conditions of lower light exposure and presented greater *WUE*, regardless of the treatment to which it was submitted.

In summary, considering that the presence/absence of BSE in leaves is a fixed character, the physiological behavior inherent to species with heterobaric and homobaric leaves were expected to *M. acutifolium* and *D. mollis*, respectively. In fact, the physiological responses to the experimental conditions were different for the studied species, which is associated with different strategies of heterobaric and homobaric leafed species for the use of water and light. Nevertheless, a lower distance between veins was expected in leaves under higher light intensity and lower water available, which was not confirmed in any of the species studied. We suggest that the maintenance of the distances between the veins immersed in the mesophyll in *M. acutifolium* and *D. mollis* reflects their adaptation to the environmental conditions of the Brazilian savanna.

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CAPÍTULO III

TAXONOMIC SIGNIFICANCE AND EVOLUTION OF HOMOBARIC AND HETEROBARIC LEAVES IN ADESMIA CLADE SPECIES (LEGUMINOSAE – PAPILIONOIDEAE)[1]

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Abstract

The presence/absence of bundle sheath extensions (BSE) in a leaf blade allows classification of the leaves as heterobaric or homobaric, respectively. The compartmentalization of the mesophyll by BSE is associated with differences in functional aspects of heterobaric and homobaric leaves, such as conduction of water and luminosity, traits related to the successful colonization of different environments by plants. Therefore, studies on the distribution of different leaf types in a taxon can provide information on ecological, systematic, physiological, and evolutionary aspects of that taxon. The Adesmia clade belongs to the legume tribe Dalbergieae (Papilionoideae, Leguminosae) and comprises five genera with a neotropical or pantropical distribution. We mapped the distribution of Adesmia clade species with either homobaric or heterobaric leaves, in a search for associations with their habit, area of occurrence, and anatomical evolution. Leaves from 75 herbaceous, subshrubby, shrubby and climbing

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[1] Part of Doctoral thesis of F. H. Palermo at Graduate Program in Plant Biology, São Paulo State University, UNESP, Institute of Biosciences.

species from different environments were obtained from national and international herbaria. Leaflet blade samples were processed according to standard techniques in plant anatomy. We found that 53.4% of the species studied presented heterobaric leaves and 46.6% presented homobaric leaves. All species of the genus *Adesmia* DC. had homobaric leaves, while all species of *Amicia* Kunth and *Nissolia* Jacq. had heterobaric leaves. In contrast, *Zornia* J.F. Gmel. and *Poiretia* Vent. presented some species with homobaric leaves and others with heterobaric leaves. All climbers studied had heterobaric leaves, while 80.9% of the herbaceous species presented homobaric leaves. For the shrubby and subshrubby species analysed, 51.5% had heterobaric leaves and 48.5% had homobaric leaves. No significant association was found between the areas of occurrence of the plants and the leaf type. Rather, the presence/absence of BSE seems to be related to plant habit and taxonomic group. Our results show that within the Adesmia clade the presence of BSE in the leaf blade possibly evolved from heterobaric leaves with the presence of extensions in the second and third order (occasionally fourth order) veins to heterobaric leaves with BSE only in second order veins, and for homobaric leaves in earlier lineages (*Zornia* and *Adesmia*).

Key-words: bundle sheath extensions, evolution, Fabaceae, habit, leaf anatomy

1. Introduction

Morphological plasticity and evolutionary adaptability in heterogeneous environments are determinants of plant performance success and natural selection (Abdusalam and Li, 2018). Leaves perform a central contribution to plant fitness and their morphology results from a balance between the requirement for efficient absorption of energy from the sun for photosynthesis and minimization of disadvantages caused by environmental stresses (Fritz et al., 2018). The morpho-anatomical traits of leaves have been shown to be extremely variable in plants (Tsukaya, 2018), varying among species, populations, and individuals, but also within the same genotype (Fritz et al, 2018).

In some plant species, parenchyma or sclerenchyma cells that make up the sheath surrounding the vascular bundles immersed in the mesophyll extend towards the epidermis of both sides of the leaf blade, forming the bundle sheath extensions - BSE. The presence or absence of BSE in plant species is an important character which allows the classification of leaves as heterobaric or homobaric, respectively (Wylie, 1952; Fahn, 1990; Terashima, 1992). In heterobaric leaves, BSE divide the leaf mesophyll into small compartments (Terashima, 1992; Liakoura et al., 2009), while in homobaric leaves the mesophyll remains homogeneous (Wylie, 1952). Studies have shown that BSE have different functions in leaves, and a role in conducting water through the leaf blade and in directing light to the innermost layers of the mesophyll have been suggested (Karabourniotis et al., 2000; Nikolopoulos et al., 2002; Liakoura et al., 2009; Buckley et al., 2011).

Differences in morphological function between homobaric and heterobaric leaves appear to be related to a plant's environment (Kenzo et al., 2007; Mendes et al., 2016). Studies suggest that species with heterobaric leaves occur preferentially in environments with a defined dry and/or cold season (Terashima, 1992), while species with homobaric leaves are dominant in warm and humid regions (Kenzo et al., 2007; Kashimura et al., 2000; Pieruschka et al., 2005). In fact, Mendes et al. (2016) observed greater abundance of plant species with heterobaric leaves in the Brazilian Cerrado compared to the wetter areas of seasonal semideciduous forest.

The distribution of species with different leaf types is also related to the vegetative stratum in which the taxon occurs. A higher abundance of heterobaric leaves was observed in the uppermost strata of vegetation, both in forest emergent species (Kenzo et al., 2007; Inoue et al., 2015) and canopy species in tropical forests (Kenzo et al., 2007; Boeger et al., 2016) and in xeric environments (Liakoura et al., 2009), while homobaric leaves appear to be more common in the lower strata of vegetation (Kenzo et al., 2007; Boeger et al., 2016). Thus, microenvironmental factors such as light intensity, temperature and humidity seem to significantly influence the spatial distribution of species with different leaf types (Kenzo et al., 2007).

The possible relationship between the distribution of homobaric and heterobaric leaves in the environment with plant habit and the evolutionary behavior of BSE in different plant groups is an aspect that deserves attention. In some plant groups, species belonging to the same genus have the same leaf type, regardless of the ecological stratum or vegetation in which they occur (Mendes et al., 2016), while in other groups species with homobaric and heterobaric leaves occur in the same genus (Kenzo et al., 2007; Boeger et al., 2016). However, no previous studies have examined the evolutionary or ecological aspects relating to different leaf types within a specific taxonomic group. Thus, the investigation of the presence or absence of BSE within a well-established botanical group should throw light on the evolutionary behaviour of BSE.

Leguminosae Juss. has a cosmopolitan distribution and is the third most speciose family of Angiosperms, with 798 genera and approximately 23,000 species distributed amongst six subfamilies (LPWG, 2017, and recent additions). It is certainly the most speciose family in Brazil and occurs in all biomes (Flora do Brasil, 2020). Several studies on the anatomy of vegetative and reproductive organs of leguminous species have enlightened systematic and evolutionary understanding of Leguminosae taxa (Lavin et al., 2001; Paulino et al., 2011; Coutinho et al., 2015; Barros and Teixeira, 2016; Silva et al., 2017; Silva et al., 2018; Rashid et al., 2019; Vargas et al., 2018; Mendes et al., 2019; Seixas et al., 2019; Bento et al., 2020; Fortuna-Perez et al., 2021).

Recent studies focusing on the Adesmia clade (Leguminosae, Papilionoideae, Dalbergieae) have highlighted the importance of anatomical characters in understanding the phylogenetic relationships among species of the group (Fortuna-Perez et al., 2021). The clade comprises five genera (*Adesmia* DC., *Amicia* Kunth, *Nissolia* Jacq., *Poiretia* Vent. and *Zornia* J.F.Gmel) and more than 360 species of varying habit, distributed pantropically, but with the main diversity in the Neotropics (Klitgaard and Lavin, 2005). Previous studies recovered *Poiretia* as sister to a clade comprising *Amicia* and *Zornia* (Lavin et al., 2001; Fortuna-Perez et al., 2013; Fortuna-Perez et al., 2021), with *Adesmia* sister to those three. *Nissolia* (including its synonym *Chaetocalyx* DC.) is sister to the other four genera and is the earliest diverging lineage of the Adesmia clade (Fortuna-Perez et al., 2021). The aim of the present study was to investigate the occurrence of BSE in leaves of taxa of the Adesmia clade and then to map the distribution of species with homobaric and heterobaric leaves in a search for associations with habit and environment.

2. Materials and methods

2.1 Plant material for leaf type classification

Samples were obtained from fully expanded leaves of 75 species belonging to the genera *Adesmia*, *Amicia*, *Nissolia*, *Poiretia* and *Zornia*, housed in national and international herbaria. Specimen collection data are presented in Table 1.

2.2 Light microscopy

Samples of the medium region of the leaflet blade were heated in deionized water until completely submersed. Then the samples were treated in KOH 2% solution at 25ºC for at least two hours or until completely submersed. Next, the material was washed in deionized water and dehydrated in an ethyl series to 70% alcohol, in which the samples were stored (Smith and Smith, 1942).

The samples were embedded in methacrylate historesin (Leica, Germany) and sectioned (to 6 µm) in a semi-automatic rotating microtome RM 2145 (Leica, Germany). The obtained sections were stained with Toluidine Blue (O'Brien et al., 1964) and permanent slides were mounted with Entelan® (Merck). The slides were analyzed using a light microscope model BX 41 (Olympus, Japan) and the results were recorded using a digital camera.

2.3 Classification of leaf types

Our classification of leaf types adopted a broad concept of homobaric and heterobaric leaves. Thus, leaves that present BSE projecting to both sides of their blade, regardless of the vein order in which they are present, are considered to be heterobaric, while those species totally lacking BSE are considered to be homobaric leaves (Terashima, 1992).

2.4 Taxon sampling for evolutionary analysis

We used the nuclear ribosomal internal transcribed spacer region (i.e., the 5.8S and flanking ITS1 and ITS2 spacers, including flanking 18S and 25S hereafter nrITS) to infer the relationships among the species within the Adesmia clade and further investigate the presence of BSE in the leaflet blades from an evolutionary perspective. We used the nrITS DNA sequence alignment generated by Fortuna-Perez et al. (2021) for 81 samples, including the outgroups *Tipuana tipu* (Benth.) Kuntze and *Pterocarpus rohrii* Vahl from the Pterocarpus clade of the Dalbergioid legumes (Lavin et al., 2001). This phylogeny was later pruned for the ancestral trait reconstruction analysis, retaining only the species for which we have anatomical leaflet data (see below). The final pruned phylogeny comprises 62 taxa, including multiple species of the five genera within the Adesmia clade.

2.5 Phylogenetic analysis and molecular dating

We ran the phylogenetic analysis under maximum likelihood (ML) and Bayesian Inference (BI) frameworks. Initially we conducted phylogenetic analyses in separate partitions representing the conserved regions 5.8S, 18S and 25S and the fast-evolving regions ITS1 and ITS2 to investigate the potential presence of nrITS pseudogenes. We assigned the partitions by comparison with the annotated nrITS sequence of *Adesmia pinnifolia* Gillies ex Hook. & Arn. available in GenBank (AF 183497.1). We checked for differences in rates of evolution in the partitioning scheme proposed with PartitionFinder2 version 2.1.1 (Lanfear et al., 2017) using PhyML version 3.0 (Guindon et al., 2010) and the greedy search algorithm (Lanfear et al., 2012).

The PartitionFinder2 analysis identified two separate partitions, one containing the fast and the other the conserved regions, each with different rates of evolution, an indication of the absence of pseudogenes. In addition, PartitionFinder2 also estimates the best fitting model of sequence evolution for each of the two partitions identified. We used IQ-TREE version 2.0.3 (Nguyen et al., 2015; Minh et al., 2020) to run the ML analysis with 1,000 bootstrap replicates with the -sampling GENESITE option and the partition model option (-p) (Chernomor et al., 2016) specified as follows: TIMEF+G for 18S, 5.8S and 25S and TRN+G for ITS1 and ITS2. We used BEAST2 version 2.6.6 (Bouckaert et al., 2019) to infer a temporally calibrated phylogeny under the BI framework. We used the results from the topology of the ML phylogeny to constrain the Bayesian phylogeny. Specifically, we constrained: (i) a clade comprising *Zornia*, *Amicia* and *Poiretia* as monophyletic, and (ii) a clade comprising *Adesmia*, *Zornia*, *Amicia* and *Poiretia* as monophyletic. We ran two separate analyses of 20x10^7 generations each, sampling trees every 2,000 generations, with the uncorrelated lognormal relaxed molecular clock model and the Yule tree prior. We used Tracer version 1.7.2 (Rambaut et al., 2018) to ensure effective sample sizes were higher than 200 for each parameter. We then combined the two sets of posterior trees with LogCombiner with a burnin of 25% in each set, resulting in a final set of 15,002 posterior trees. We generated a Maximum clade Credibility Tree with node heights as mean heights using TreeAnnotator version 2.6.6.

We calibrated the phylogeny with three secondary calibrations following Fortuna-Perez et al. (2013) and Lavin et al. (2005): a clade comprising all 81 accessions, i.e., equivalent to the root node of the outgroups and ingroup of this study, with a mean age of 35.3 million years (Ma) and a standard deviation of 2.3; a clade comprising *Poiretia*, *Amicia* and *Zornia* with mean age of 16.1 Ma, offset to 10.8 Ma and a standard deviation of 1.9; and the crown node of *Nissolia* with a mean age of 8.5 Ma and a standard deviation of 1.4. All calibrations were assigned with a normal distribution.

2.6 Ancestral State Reconstruction (ASR)

The ancestral states were coded according to the presence or absence of bundle sheath extensions (BSE) in the leaflet blade. We used three states for these anatomical characters (0: homobaric leaflets, i.e., BSE absent; 1: heterobaric leaflets with BSE only in the secondary veins (large heterobaric); 2: heterobaric leaflets with BSE in secondary, tertiary, and quaternary veins (typical heterobaric).

The ancestral states of the leaflet morphological characters coded were reconstructed using stochastic character mapping (Huelsenbeck et al., 2003), a Bayesian-based approach implemented in the R package Phytools version 1.0.1 (Revell, 2012). Initially we estimated ancestral states under three different models: equal rates (ER), symmetric rates (SYM) and all-rates different (ARD). The best fitting model, SYM, was determined with the Akaike Information Criterion (AIC). For the stochastic character mapping we randomly sampled 100 posterior trees from the BI analysis output and generated 1,000 stochastic character maps. One random stochastic character map was then plotted on a consensus phylogenetic tree with pie charts representing the ancestral state posterior probabilities inferred for each node. A complete list of vouchers and Genbank accession numbers is provided in Appendix A.

2.7 Statistical analysis

The distribution of species with homobaric and heterobaric leaves in different environments was analyzed only for species occurring in Brazil in the following environments: Amazon Forest, Caatinga, Cerrado, Atlantic Forest, and Pampa. Furthermore, the association between leaf types and plant habit was analyzed for all studied species. The Chi-square test was used for both statistical analyses and was performed using the Sigmaplot 12.0 program to test the independence between groups; the significance level adopted was $p < 0.05$.

3. Results

Of the 75 species of the Adesmia clade studied, 40 (53.4%) had heterobaric leaves and 35 (46.6%) had homobaric leaves (Table 2). Of the species with heterobaric leaves, 24 (60%)

presented BSE in secondary, tertiary and quaternary veins, while 16 (40%) had BSE only in the second order veins.

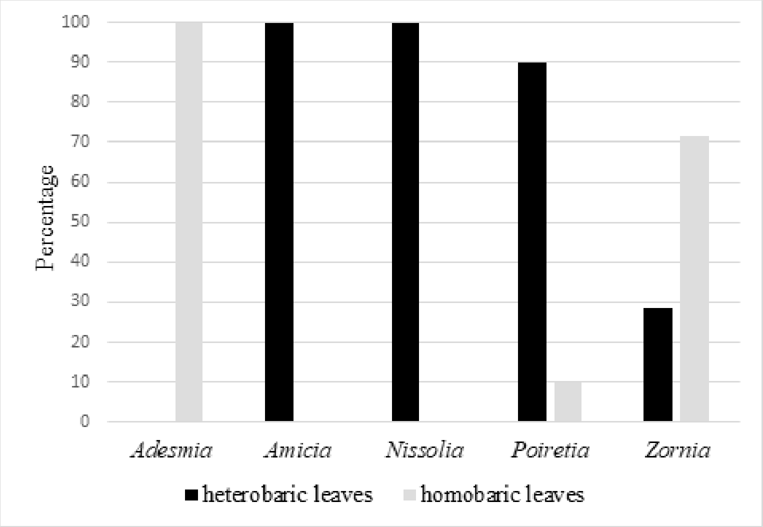

Fig. 1. Percentage of species with heterobaric and homobaric leaves in the Adesmia clade.

Regarding the distribution of species with different leaf types, the genera studied presented different proportions of homobaric and heterobaric species (Fig. 1; Table 2). All species of *Adesmia* had homobaric leaves (Fig. 2a), while all species of *Amicia* (Fig. 2b, c) and *Nissolia* (Fig. 2d, e) had heterobaric leaves (Table 2). The *Amicia* species differed from each other in the order of the veins that presented BSE (Table 2), with species with leaves containing BSE only in the second order veins (Fig. 2b) and species with leaves with BSE in the second and third orders veins (Fig. 2c). Most of the studied species (85%) of *Nissolia* presented leaves with BSE in the second and third order veins (Fig. 2d; Table 2), and just four species presented BSE only in the second order veins (Fig. 2e; Table 2).

Of the studied species of *Poiretia*, 90% had heterobaric leaves (Table 2). Among these, four species had BSE in the second order veins (Fig. 2f), while three species had BSE in the second and third order veins (Fig. 2g) and two species had BSE in the second, third and fourth order veins (Fig. 2h). *Poiretia longipes* differed in having homobaric leaflets (Fig. 2i). In *Zornia*, 76.9% of the studied species had homobaric leaves (Fig. 2j). Of the species that presented heterobaric leaves, most had BSE only in the second order veins (Fig. 2k; Table 2).

Regarding cell composition, the vast majority (92.5%) of species with heterobaric leaves had BSE consisting exclusively of parenchyma cells (Fig. 2b, d-f, k). However, some species of *Amicia* (Fig. 2c) and *Poiretia* (Fig. 2g, h) had mixed BSE composed of parenchyma and sclerenchyma cells (Table 2).

The species of the Adesmia clade studied ranged in habit from herbaceous, to subshrubby, shrubby and climbing (Table 2). Data analysis revealed a significant association between the species habit and the leaflet type (χ^2 = 31,996; d.f = 2; p = <0,001; N= 70; Table 3).

Table 3. Distribution of species with homobaric and heterobaric leaves in the Adesmia clade related to species habit. (Qui-square test χ^2 = 28,905; d.f = 2; p = <0,001)

Habit	Nº of species	Leaf type	
		Homobaric	**Heterobaric**
Vines	21	0 (0%)	21 (100%)
Shrub/ Subshrub	54	35 (64,8%)	19 (35,2%)

The shrubs and subshrubs of the Adesmia clade had a greater abundance of homobaric leaves (64,8%). In contrast, all climbing species analyzed had heterobaric leaves.

Species occurring on different continents and different types of vegetation were sampled (Table 2), but no significant association was found between the leaf type, vegetation and geography (χ^2 = 9,5444; d.f = 4; p = <0,049; N = 97).

The crown node age of the Adesmia clade is estimated to have diversified about 31.66 Ma (26.88-36.19 [95% Highest Posterior Density HPD]) (Fig. 3). The diversification of *Poiretia* is estimated at 14.81 Ma (9.77-19.92 [95% hPD]) and *Nissolia* at 10.40 Ma (8.28-12.55 [95% HPD]) (Fig. 3). *Amicia* diverged about 9.83 Ma (5.16-15.05 [95% HPD]) and *Adesmia* 11.36 Ma (7.20-16.12 [95% HPD]), while *Zornia* diversified about 8.43 Ma (Fig. 3).

Considering the ancestral state reconstruction analysis, the hypothetical ancestor of *Adesmia* probably had homobaric leaves (Fig. 4), and all species studied from this genus retained this ancestral state. The same applies to *Zornia*, with the majority of species retaining homobaric leaves, although *Z. reptans*, *Z. gibbosa*, *Z. crinita*, *Z. latifolia* and *Z. mitziana* had heterobaric leaves with BSE only in their secondary order veins; and *Zornia pardina* had heterobaric leaves with BSE in its secondary and tertiary veins. In *Nissolia* we observed that its hypothetical ancestor most likely had heterobaric leaves, although only *Nissolia klugii* and *Nissolia subulatus* had heterobaric leaves with BSE confined to the secondary order veins.

Our results suggest that the hypothetical ancestor of *Amicia* had heterobaric leaves with BSE restricted to secondary order veins. Of the four species studied, only *Amicia lobbiana* presented BSE in second and third order veins. The hypothetical ancestor of *Poiretia* might have had homobaric or heterobaric leaves.

4. Discussion and conclusions

Our results show that Adesmia clade species have different leaf types regarding the presence/absence of BSE. Differences were also found in the distribution of BSE in the leaf blade, these either having BSE only around the second order veins, or in the second and third order veins, or in the second, third and fourth order veins. According to Rodrigues et al. (2017), species with leaves that present BSE only around the second order veins (also referred to as *larger heterobaric*), have gas exchange rates similar to those of leaves that have BSE around the smaller veins (*typical heterobaric*). In contrast, leaves with an absence of BSE (*typical homobaric*) have photosynthetic rates closer to those of species with leaves containing BSE that extend only to one side of the leaflet blade leading to incomplete compartmentalization of the mesophyll (*semi-heterobaric*). In our study, considering the functionality of the mesophyll compartmentalization, heterobaric leaves are considered to have complete compartmentalization of the mesophyll, regardless of the size of the compartments formed (*typical heterobaric* and *larger heterobaric*). Leaves with homogenous mesophyll devoid of compartmentalization were considered homobaric.

Although the relationship between leaf type and vegetation has been reported in several plant studies (Kenzo et al., 2007; Zsögön et al., 2015; Mendes et al., 2016), the analysis of the distribution of species with homobaric and heterobaric leaves in the Adesmia clade did not show a significant association between leaf type and environment. Our results do show, however, that the occurrence of heterobaric and homobaric leaves appears to be related to plant habit. In our study, we observed that all climbing species, regardless of the genus to which they belong, had heterobaric leaves. Climbing species associate themselves with larger plants in a search for higher levels of light (Kenzo et al., 2007). In these species, the presence of BSE might be associated with a better use of solar radiation, since the BSE cells can act as light conducting channels for the innermost layers of the mesophyll (Karabourniotis et al., 2000), optimizing the conditions for photosynthesis. Furthermore, the presence of BSE may be related to water use efficiency by heterobaric species, facilitating water transport through the mesophyll (Terashima, 1992; Scoffoni et al., 2008). In addition, it might prevent dehydration of the leaf blade (Wylie, 1943, 1951, 1952) and assist in closing stomata in response to high temperatures (Terashima, 1992).

The absence of BSE in homobaric leaves would guarantee a greater area of leaf mesophyll occupied by cells with photosynthetic pigments (Terashima, 1992). Furthermore, in humid environments such as vegetation understory, the lack of compartmentalization of the mesophyll by BSE in homobaric leaves would allow a more efficient lateral diffusion of gases, helping to maintain higher photosynthesis rates (Pieruschka et al., 2005; Liakoura et al., 2009; Barbosa et al., 2019).

Regarding the shrubby and subshrubby species of the Adesmia clade, the number of taxa with homobaric leaves is equivalent to those with heterobaric leaves. Given that shrubby and subshrubby species most frequently occur in the intermediate strata of vegetation, the microclimatic conditions in light intensity (Boeger et al., 2016) and air humidity typical of this stratum could favour the occurrence of species with homobaric leaves due to their greater area of photosynthetic activity in the mesophyll (Kenzo et al., 2007), and species with heterobaric leaves due to their greater efficiency in water use (Terashima, 1992; Scoffoni et al., 2008).

In most heterobaric species the BSE is made up of exclusively parenchyma cells, except for four shrubby and/or subshrubby species belonging to *Poiretia* and *Amicia*. Bundle sheath extensions consisting of parenchyma and sclerenchyma cells were observed in *A. lobbiana*, *P. coriifolia* and *P. latifolia*. It therefore appears that in these species the compartmentalization of the mesophyll is a result of cells in the bundle sheath that can either maintain their pectocellulosic walls or become lignified. Moreover, in *P. marginata* and *P. coriifolia* where the BSE were enlarged in the second and third order veins, the involvement of the pericycle in the mesophyll compartment is evident. The contribution of the pericycle in the formation of the cell extensions that compartmentalize the mesophyll seems to be a common aspect and has been described for plants belonging to other families (Menezes et al., 2006). The presence of lignified cells around vascular bundles can provide greater support for the leaf/leaflet blade (Aoki et al., 1978; Boeger et al., 2016) and protection against herbivory (Scatena and Dias, 2006).

Species with heterobaric leaves are predominant in the studied group, although distribution of leaf types differed among the genera of the Adesmia clade. Although studies show that most species belonging to a given taxonomic group have the same leaf type regarding the presence/absence of BSE, the occurrence of species with homobaric and heterobaric leaves has been recorded in the same family or genus (Kenzo et al., 2007; Mendes et al., 2016). Nevertheless, most of the sampled genera showed leaf type homogeneity among the studied species.

All representatives of the genus *Adesmia* had homobaric leaves, while all species of *Amicia* and *Nissolia* had heterobaric leaves. In contrast, *Zornia* and *Poiretia* had species with homobaric leaves and others with heterobaric leaves (Fig. 1, 3). Phylogenetically, *Zornia* is resolved in a clade together with *Amicia* and *Poiretia,* as sister to *Amicia* (Fortuna-Perez et al., 2021), although in a previous study by Fortuna-Perez et al. (2013) *Zornia* had appeared as sister to *Poiretia*. It is noteworthy that all species of *Nissolia* and *Amicia* have only heterobaric leaves, showing that this is an additional unifying characteristic of these two genera. *Adesmia* was the only genus within the clade that presented only homobaric leaves, a diagnostic characteristic for the genus.

Thus, from a phylogenetic perspective, our results show that the presence/absence of BSE in the Adesmia clade has evolved in several independent lineages, and could have several independent origins. The ancestral state reconstruction analysis did not identify the BSE character state of the ancestor of the Adesmia clade, so an analysis which includes a greater number of species belonging to the group may be necessary to determine this. Nevertheless, for the studied genera, we observed that the possible ancestor had different characteristics.

The ancestor of *Nissolia* and *Poiretia* most likely had heterobaric leaves with BSE in the second and third order veins (Fortuna-Perez et al., 2013); these two genera emerged 10 million and 14 million years ago, respectively. Although *Zornia* and *Amicia* are sister genera, the ancestors of each genus possibly presented different anatomical characteristics regarding the presence of BSE, as the ancestor of *Amicia* possibly had heterobaric leaves with BSE only in the secondary veins, while the possible ancestor of *Zornia* lacked BSE. This is possibly explained by the environmental predilection of the two genera, with *Zornia* preferring cerrado and caatinga vegetation where high temperatures predominate, and *Amicia* growing in the Andes with lower temperatures. As mentioned earlier, plants with heterobaric leaves occur preferentially in environments with a defined dry and/or cold season (Terashima, 1992), while species with homobaric leaves are dominant in warm and humid regions (Kenzo et al., 2007; Kashimura et al., 2000; Pieruschka et al., 2005).

For *Adesmia* and *Zornia* their probable ancestor had homobaric leaves, a characteristic that is retained in almost all species of these genera, except in a few species of *Zornia*. *Zornia crinita* and *Z. pardina* occur in Central and South America, while *Z. gibbosa* and *Z. reptans* occur in Asia and Africa, respectively; *Z. mitziana* is endemic to Brazilian cerrado, and *Z. latifolia* is the most widely distributed species, occurring both in the Americas and in Africa. All these species have a preference for dry environments with high temperatures. We thus found no relationship between the leaf type and vegetation type.

According to our results, we can infer that within the Adesmia clade, BSE presence in the leaflet blade appears to have evolved from heterobaric leaves (in *Poiretia*) with bundle sheath extensions in the second order veins to homobaric leaves in *Adesmia*; followed by an apparent reversal to heterobaric leaves (in *Nissolia*) with the presence of extensions in the second and third (occasionally fourth) order veins. Later, in *Amicia* heterobaric leaves evolved with BSE only in second order veins. In the earlier *Zornia* lineage homobaric leaves predominate.

In conclusion, our results present a wide diversity of leaflet types in the Adesmia clade with regard to occurrence, distribution, and structure of BSE. Our data point to homobaric and heterobaric leaves appearing at different times during the evolution of the group. The presence of BSE (heterobaric leaves) is a unifying characteristic of the genera *Amicia* and *Nissolia*. In addition, the absence of BSE is a unifying characteristic of the genus *Adesmia.* Almost all genera of the Adesmia clade have representatives with heterobaric leaves (except *Adesmia*), showing that heterobaric leaf evolution was an important trait for the establishment of species of these genera in dry, open environments with a strong incidence of light.

Although we have not found a relationship between vegetation type and the presence/absence of BSE, their presence or absence does appear to be related to microenvironmental factors such as light intensity, water availability and temperature, as well as to plant habit.

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Appendix A. Voucher information for the sequences used in this study.

Taxon name, country and/or locality, collector(s), collection number and herbarium, and GenBank accession numbers available (ITS).

Adesmia araujoi Burkart, Brazil, Rio Grande do Sul, *Valls et al. 9855* (UEC); MT663730. *Adesmia arillata* Miotto, Brazil, Paraná, *Valls et al. 11361* (UEC); MT663729. *Adesmia bicolor* (Poir.) DC., Brazil, Rio Grande do Sul, *Valls et al. 9614* (UEC); MT663728. *Adesmia cilliata* Vogel, Brazil, Rio Grande do Sul, *Valls et al. 10809* (UEC); MT663727. *Adesmia incana* Vogel, Brazil, Rio Grande do Sul, *Stehmann et al. 2046* (UEC); MT663726. *Adesmia latifolia* (Spreng.) Vogel, Brazil, Rio Grande do Sul, *Vales et al. 10004* (UEC); MT663725. *Adesmia muricata* var. *muricata* (Jacq.) DC., Brazil, Rio Grande do Sul, *Vales et al. 10289* (UEC); MT663724. *Adesmia punctata* var. *punctata* (Poir.) DC., Brazil, Rio Grande do Sul, *Vales et al. 9996* (UEC); MT663723. *Adesmia reitziana* Burkart, Brazil, Santa Catarina, *Vales et al. 8045* (UEC); MT663731. *Adesmia rocinhensis* Burkart, Brazil, Rio Grande do Sul, *Vales et al. 10805* (UEC); MT663732. *Adesmia securigerifolia* Herter, Brazil, Paraná, *Vales et al. 10284* (UEC); MT663722. *Adesmia tristis* Vogel, Brazil, Santa Catarina, *Vales et al. 10555* (UEC); MT663733. *Amicia andicola* (Griseb.) Harms, Bolívia, Tarija, *Sarkinen et al. 2138* (FHO); KF477885. *Amicia fimbriata* Harms ex Kuntze, Argentina, *A. Schiminii & R. Vanii 22492* (ICN); MT663738. *Amicia lobbiana* Benth., Bolívia, La Paz, *Sarkinen et al. 2057* (FHO); KF477886. *Amicia medicaginea* Griseb., Bolívia, Chuquisaca, *Sarkinen et. al. 2115* (FHO); KF477887. *Nissolia bracteosa* (Rudd) T.M.Moura & Fort.-Perez, Brazil, Pará, *Ribeiro et al. 1255* (INPA); MT663735. *Nissolia brasiliensis* (Vogel) T.M.Moura & Fort.-Perez, Brazil, Bahia, *Fortuna-Perez et al. 191* (UEC); KF477888. *Nissolia klugii* (Rudd) T.M.Moura & Fort.-Perez, Brazil, Acre, *Daly 6778* (NY); AF183506. *Nissolia latisiliqua* (Poir.) T.M. Moura & Fort.-Perez, Equador, El Oro, *Conejo et al. 4063* (K); MT663736. *Nissolia longiflora* (A. Gray) T.M.Moura & Fort.-Perez, Brazil, Minas Gerais, *Mello Barreto 5704* (F); KF477889. *Nissolia microptera* (L.) T.M.Moura & Fort.-Perez, Brazil, Bahia, *Fortuna-Perez et al. 313* (UEC); MT663734. *Nissolia nigricans* (Burkart) T.M.Moura & Fort.-Perez, Argentina, Misiones, *Vanni 2955* (F); AF183508. *Nissolia subulata* (Makinder) I. Castro, Fort.-Perez & G.P. Lewis, Brazil, Bahia, *L.P. Queiroz et al. 5294.* (K); MT663737. *Nissolia vincentina* (L.) T.M.Moura & Fort.-Perez, Brazil, Bahia, *Fortuna-Perez et al. 313* (UEC); KF477490. *Nissolia leiogyne* Sandwith, México, Jalisco, *Magallanes 2902* (F); AY253878. *Nissolia schottii* A. Gray, México, Sonora, *Joyal 2094* (NY); AF183510. *Poiretia angustifolia* Vogel, Brazil, Goiás, *Fonseca et al. 1419* (MO); AF1853503. *Poiretia bahiana* Cl. Müll., Brazil, Bahia, *Fortuna-Perez et al. 179* (UEC); KF477891. *Poiretia longipes* Harms, Brazil, Mato Grosso do Sul, *Pott et al. 11613* (UEC); MT663739. *Poiretia marginata* Cl. Müll., Brazil, Minas Gerais, *Schiavini 87* (UEC); MT663740. *Poiretia punctata* (Willd.) Desv., Brazil, Bahia, *Fortuna-Perez et al. 203* (UEC); KF477892. *Poiretia tetraphylla* (Poir.) Burkart, Brazil, Rio Grande do Sul, *Stehmann et al. 1866* (UEC); MT663741. *Pterocarpus rohrii* Vahl, Brazil, Amazonas, *Sothers 1025* (SP); EF451061. *Tipuana tipu* (Benth.) Kuntze, Argentina, *Lavin 5796* (MONT); AF189056. *Zornia brasiliensis* Vogel, Brazil, Bahia, *Fortuna-Perez et al. 285* (UEC); KF477896. *Zornia cearensis* Huber, Brazil, Ceará, *Coradin et al. 7848* (M); KF477898. *Zornia confusa* Vanni, Brazil, Minas Gerais, *Fortuna-Perez et al. 115* (UEC); KF477899. *Zornia crinita* (Mohlenbr.) Vanni, Brazil, Minas Gerais, *Semir et al. 7255* (UEC); KF477930. *Zornia cryptantha* Arechav., Brazil, Minas Gerais, *Fortuna-Perez et al. 457* (UEC); KF477901. *Zornia curvata* Mohlenbr., Brazil, São Paulo, *Fortuna-Perez et al. 323* (UEC); KF477902. *Zornia dyctiocarpa* DC., Australia, *Kaspiev 166* (G); KF477903. *Zornia echinocarpa* Benth., Brazil, Bahia, *Fortuna-Perez et al. 301* (UEC); KF477904. *Zornia flemmingioides* Moric., Brazil, Bahia, *Fortuna-Perez et al. 167* (UEC); KF477905. Zornia *gardneriana* Moric., Brazil, Piauí, *Gardner 2102* (G); KF477927. *Zornia gibbosa* Span., India, *Pant 43208* (G); KF477906.

Zornia laevis Schltdl. & Cham., Mexico, *Karminski s.n.* (M); KF477909. *Zornia latifolia* Sm., Brazil, São Paulo, *Fortuna-Perez 66* (UEC); KF477910. *Zornia leptophylla* (Benth.) Pittier, Brazil, Pernambuco, *Heringer et al. 196* (PEUFR); KF477911. *Zornia milneana* Mohlenbr., South Africa, *Gilmore 1970* (G); KF477912. *Zornia mitziana* Costa, Brazil, Goiás, *Fortuna-Perez et al. 216* (UEC); KF477913. *Zornia myriadena* Benth., Brazil, Bahia, *Fortuna-Perez et al. 190* (UEC); KF477914. *Zornia pardina* Mohlenbr., Brazil, Minas Gerais, *Fortuna-Perez et al.* 118 (UEC); KF477915. *Zornia piurensis* Mohlenbr., Ecuador, Guayaquil, *Asplund 16008* (G); KF477916. *Zornia reptans* Harms, Tanzania, Polhill *1611* (BM); KF477917; *Zornia reticulata* Sm., Brazil, São Paulo, *Fortuna-Perez 326* (UEC); KF477918. *Zornia sericea* Moric., Brazil, Bahia, *Fortuna-Perez et al. 274* (UEC); KF477919. *Zornia setosa* Bak. f., Kenya, *Maas 6171* (G); KF477920. *Zornia subsessilis* Fort.-Perez & A.M.G. Azevedo, Brazil, Minas Gerais, *Fortuna-Perez et al. 107* (UEC); KF477921. *Zornia tenuifolia* Moric., Brazil, Bahia, *Fortuna-Perez et al. 275* (UEC); KF477922. *Zornia venosa* Mohlenbr., Mexico, Muller *3397* (MO); KF477931. *Zornia virgata* Moric., Brazil, Minas Gerais, *Fortuna-Perez et al. 428* (UEC); KF477926.

Figures and legends

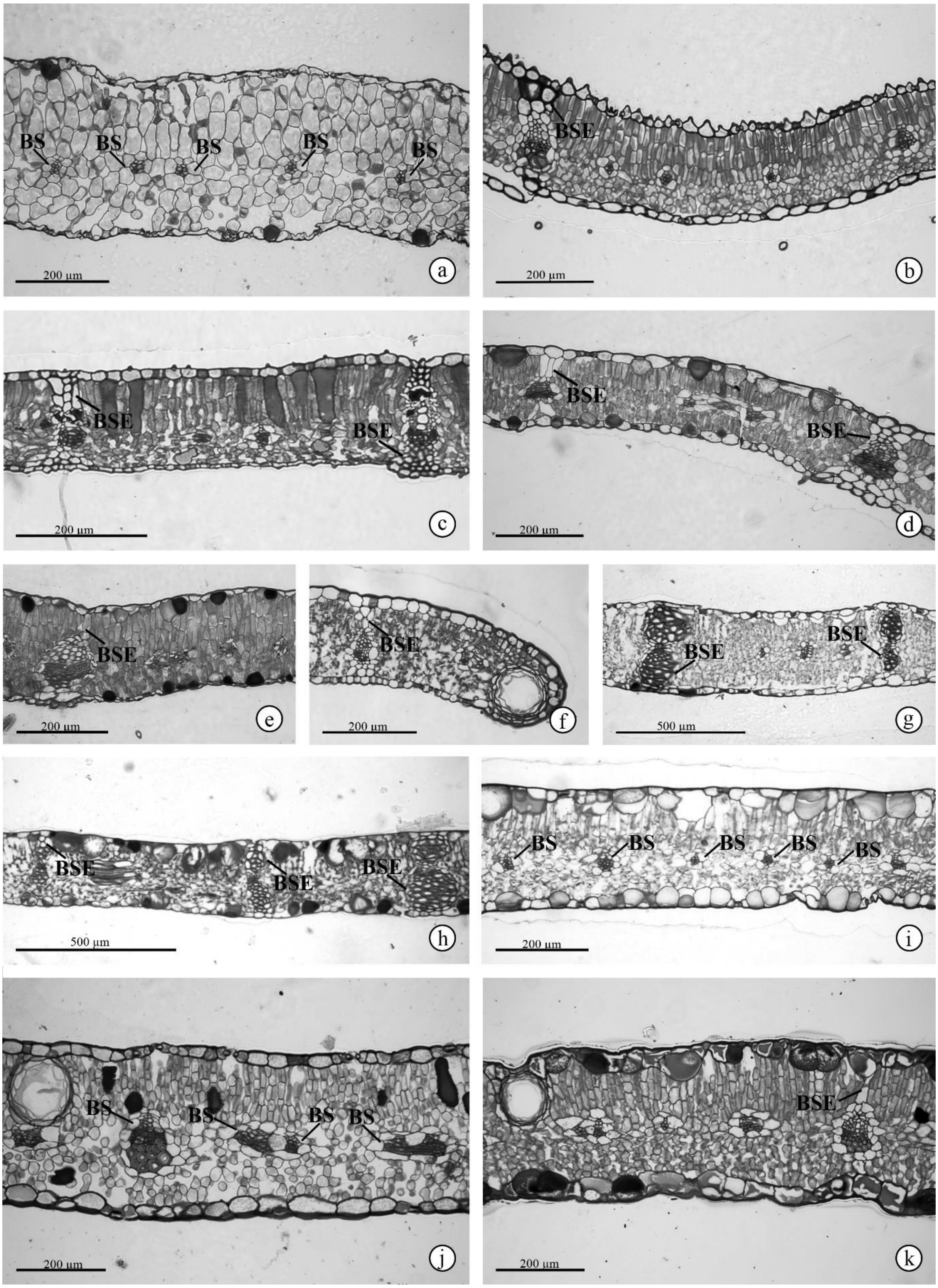


Fig. 2. Cross sections of leaflet blades of Adesmia clade species. (a, i, j) Homobaric leaves. (b-h, k) Heterobaric leaves (a) *Adesmia securigerifolia*. (b) *Amicia andicola*. (c) *Amicia lobbiana*. (d) *Nissolia wislizenii*. (e) *Nissolia gentryi*. (f) *Poiretia angustifolia*. (g) *Poiretia marginata*. (h) *Poiretia latifolia*. (i) *Poiretia longipes*. (j) *Zornia reptans*. (k) *Zornia flemmingioides*. BSE = bundle sheath extensions. BS = bundle sheath.

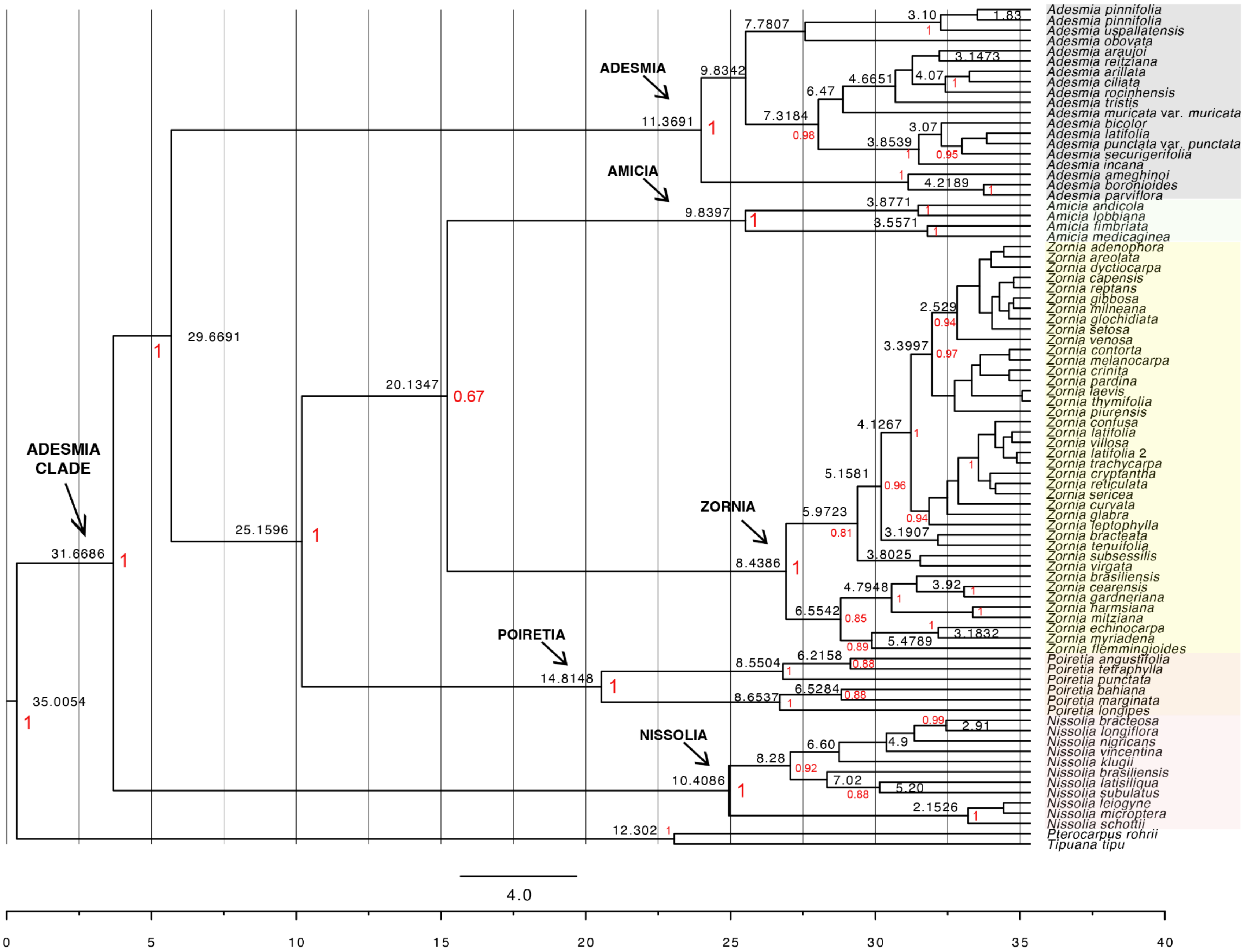
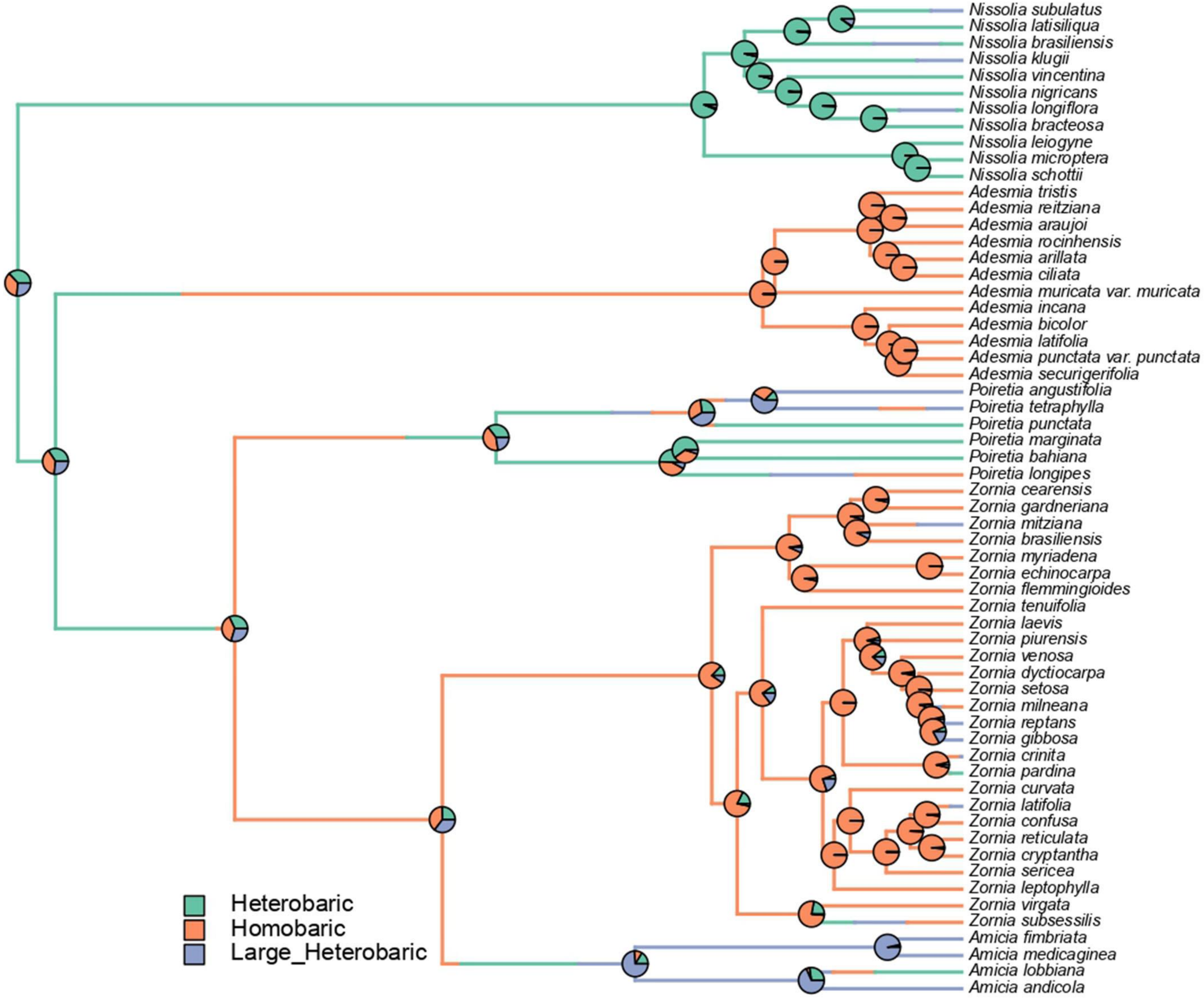

ADESMIA
AMICIA
ZORNIA
POIRETIA
NISSOLIA
ADESMIA CLADE
35.0054
1
31.6686
1
29.6691
1
25.1596
1
20.1347
0.67
14.8148
1
11.3691
1
9.8342
7.7807
3.10
1.83
1
3.1473
4.07
1
4.6651
6.47
7.3184
0.98
3.07
3.8539
1
0.95
1
4.2189
1
9.8397
1
3.8771
1
3.5571
1
2.529
0.94
0.97
3.3997
4.1267
1
0.96
5.1581
1
0.94
5.9723
0.81
3.1907
3.8025
8.4386
1
4.7948
3.92
1
1
1
6.5542
0.85
1
3.1832
0.89
5.4789
8.5504
6.2158
0.88
1
6.5284
0.88
8.6537
1
10.4086
1
8.28
0.92
6.60
4.9
0.99
2.91
7.02
0.88
5.20
2.1526
1
12.302
1
Adesmia pinnifolia
Adesmia pinnifolia
Adesmia uspallatensis
Adesmia obovata
Adesmia araujoi
Adesmia reitziana
Adesmia arillata
Adesmia ciliata
Adesmia rocinhensis
Adesmia tristis
Adesmia muricata var. muricata
Adesmia bicolor
Adesmia latifolia
Adesmia punctata var. punctata
Adesmia securigerifolia
Adesmia incana
Adesmia ameghinoi
Adesmia boronioides
Adesmia parviflora
Amicia andicola
Amicia lobbiana
Amicia fimbriata
Amicia medicaginea
Zornia adenophora
Zornia areolata
Zornia dyctiocarpa
Zornia capensis
Zornia reptans
Zornia gibbosa
Zornia milneana
Zornia glochidiata
Zornia setosa
Zornia venosa
Zornia contorta
Zornia melanocarpa
Zornia crinita
Zornia pardina
Zornia laevis
Zornia thymifolia
Zornia piurensis
Zornia confusa
Zornia latifolia
Zornia villosa
Zornia latifolia 2
Zornia trachycarpa
Zornia cryptantha
Zornia reticulata
Zornia sericea
Zornia curvata
Zornia glabra
Zornia leptophylla
Zornia bracteata
Zornia tenuifolia
Zornia subsessilis
Zornia virgata
Zornia brasiliensis
Zornia cearensis
Zornia gardneriana
Zornia harmsiana
Zornia mitziana
Zornia echinocarpa
Zornia myriadena
Zornia flemmingioides
Poiretia angustifolia
Poiretia tetraphylla
Poiretia punctata
Poiretia bahiana
Poiretia marginata
Poiretia longipes
Nissolia bracteosa
Nissolia longiflora
Nissolia nigricans
Nissolia vincentina
Nissolia klugii
Nissolia brasiliensis
Nissolia latisiliqua
Nissolia subulatus
Nissolia leiogyne
Nissolia microptera
Nissolia schottii
Pterocarpus rohrii
Tipuana tipu
4.0
0
5
10
15
20
25
30
35
40

Fig. 3.| Maximum clade credibility tree resulting from BEAST2 analysis of nuclear ribosomal ITS sequence data sets for the Adesmia clade using uncorrelated lognormal relaxed molecular clock model and Yule tree prior. Values above branches represent nodes' ages reported in million years as mean ages. Values in red represent posterior probabilities.


Nissolia subulatus
Nissolia latisiliqua
Nissolia brasiliensis
Nissolia klugii
Nissolia vincentina
Nissolia nigricans
Nissolia longiflora
Nissolia bracteosa
Nissolia leiogyne
Nissolia microptera
Nissolia schottii
Adesmia tristis
Adesmia reitziana
Adesmia araujoi
Adesmia rocinhensis
Adesmia arillata
Adesmia ciliata
Adesmia muricata var. muricata
Adesmia incana
Adesmia bicolor
Adesmia latifolia
Adesmia punctata var. punctata
Adesmia securigerifolia
Poiretia angustifolia
Poiretia tetraphylla
Poiretia punctata
Poiretia marginata
Poiretia bahiana
Poiretia longipes
Zornia cearensis
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Zornia flemmingioides
Zornia tenuifolia
Zornia laevis
Zornia piurensis
Zornia venosa
Zornia dyctiocarpa
Zornia setosa
Zornia milneana
Zornia reptans
Zornia gibbosa
Zornia crinita
Zornia pardina
Zornia curvata
Zornia latifolia
Zornia confusa
Zornia reticulata
Zornia cryptantha
Zornia sericea
Zornia leptophylla
Zornia virgata
Zornia subsessilis
Amicia fimbriata
Amicia medicaginea
Amicia lobbiana
Amicia andicola
🟩 Heterobaric
🟧 Homobaric
🟦 Large_Heterobaric

Fig. 4. Ancestral state reconstruction of the Adesmia clade using stochastic character mapping implemented in Phytools. Leaf blades were coded for the presence and absence of bundle sheath extensions (BSE) as follows: homobaric leaves devoid of BSE (orange); heterobaric leaves with BSE only in secondary veins (purple) and heterobaric leaves with BSE in secondary, tertiary, and quaternary veins (green). Pie charts represent the ancestral state posterior probabilities inferred for each node.

Tables

Table 1. List of Adesmia clade species (Leguminosae-Papilionoideae) studied and voucher information.

Species	Collection place	Date	Collector's name /Nº of collector	Herbarium
Adesmia araujoi Burkart	Bom Jesus, RS, Brasil	10/12/1986	Vales/10801	UEC
Adesmia arillata Miotto	Ponte serrada, SC, Brasil	20/11/1986	Vales, Boldrini, Zanata/10565	UEC
Adesmia bicolor (Poir.) DC.	São Borja, RS, Brasil	03/12/1986	Gonçalves, Vales, Moraes e Boldrini/10669	UEC
Adesmia ciliata Vogel	Bom Jesus RS, Brasil	07/12/1986	Gomes Vales e Miotto/10807	UEC
Adesmia incana Volgel	Santana do Livramento, RS, Brasil	03/12/1985	Vales, Gonzaga, Lemos e Boechat/9636	UEC
Adesmia latifolia (Spreng.) Vogel	Urubici, SC, Brasil	24/11/1984	Vales, Dall'Agnol, Gomes e Miotto/8049	UEC
Adesmia muricata var muricata Gillies ex Hook. & Arn.	Caçapava do sul, RS, Brasil	06/09/1986	Vales e Silva/ n/a	BOTU
Adesmia punctata var hilariana Benth.	Vacaria, RS, Brasil	n/a	Vales, Gomes e Miotto/10812	UEC
Adesmia punctata var punctata (Poir.) DC.	Bagé, RS, Brasil	29/11/1985	Vales, Gomes, Miotto e Boldrini/9610	UEC
Adesmia reitziana Burkart	Urubici, SC, Brasil	24/11/1984	Vales, Dall'agnol, Gomes e Miotto/8045	UEC
Adesmia rocinhensis Burkart	Bom Jesus, RS, Brasil	10/12/1986	Vales, Gomes, Miotto/10808	UEC
Adesmia securigerifolia Vogel	Bagé, RS, Brasil	06/09/1986	Vales, Gerardi, Deiro, Silva/10283	UEC
Adesmia tristis Volgel	Vacaria, RS, Brasil	09/12/1986	Vales, Gomes e Miotto/10766	UEC
Amicia andicola (Griseb.) Harms	Chuquisaca, Bolívia	31/01/2007	TE Särkinen *et al.*/2118	MO
Amicia fimbriata Harms ex Kuntze	Prov. de Jujuy, Argentina	29/03/1977	A Krapovickas & Schinini/30708	CTES
Amicia lobbiana Benth. ex Rusby	Depto. Puno, Peru	21/11/1938	C Vargas/9656	K
Amicia medicaginea Griseb.	Tucumán, Argentina	09/02/1907	Lillo/5760	MBM
Nissolia wislizeni (A. Gray) A. Gray	Chihuahua, México	08/1976	A Enriques / n/a	NY
Nissolia acutifolia (Vogel) T.M. Moura & Fort.-Perez	Gruta da imprensa, DF, Brasil	19/02/1960	AP Duarte/5191	NY
Nissolia blanchetiana (Benth.) T.M. Moura & Fort.-Perez	Macaúbas, BA, Brasil	15/06/2004	G Hatschbach; M Hatschbach; OS Ribas/77829	CESJ
Nissolia bracteosa (Rudd) T.M. Moura & Fort.-Perez	Formoso do Rio Preto, BA, Brasil	03/05/2009	D Cardoso *et. al.*/2646	HUEFS
Nissolia brasiliensis (Vogel) T.M. Moura & Fort.-Perez	Andaraí, BA, Brasil	15/03/2007	AP Fortuna-Perez/171	BOTU

Nissolia chacoensis (Vanni) T.M. Moura & Fort.-Perez	Sta. cruz de la sierra, Bolívia	12/02/1998	A Fuentes & G Navarro/2283	MO
Nissolia chiapensis Rudd.	Chiapas, México	07/11/1981	DE Breedlove/55042	NY
Nissolia fruticosa Jacq.	Sena Madureira, Acre	04/09/1995	ARS Oliveira *et al.*/636	NY
Nissolia gentryi Rudd.	Sonora, México	17/03/1998	AL Reina *et al.*/98-270	NY
Nissolia klugii (Rudd) T.M. Moura & Fort.-Perez	Sta. Rosa, Peru	11/08/2007	R Vaquez/32698	K
Nissolia latisiliqua (Poir.) T.M. Moura & Fort.-Perez	Santo Domingo de los Colorados, Ecuador	02/11/1961	PCD Cazalet & TD Pennington/5252	US
Nissolia laxior (B.L. Rob.) Rose	Oaxaca, México	10/06/1985	R Torres C e A Gardia M./6747	NY
Nissolia leiogyne Sandwith	Colima, México	21/09/1958	RM Vaugh/18052	s/i
Nissolia longiflora (Benth. ex A. Gray) T.M. Moura & Fort.-Perez	Sta. cruz de la sierra, Bolivia	17/05/2005	MH Nee Nee/53044	MO
Nissolia microptera Poir.	Sonora, México	19/08/1994	P Jenkins *et al.*/94-68	s/i
Nissolia nigricans (Burkart) T.M. Moura & Fort.-Perez	Missiones, Argentina	17/12/1991	RO Vanni; M Ferrucci; GL Vanni/2955	MO
Nissolia platycalyx S. Watson	Puente Chorro, México	16/05/1073	ME Johnston *et al.*/11028	s/i
Nissolia platycarpa Benth.	Nuevo Leon, México	22/10/1963	HD Ripley; RC Barneby/13257	s/i
Nissolia vincentina (Ker Gawl.) T.M. Moura & Fort.-Perez	Catolés, BA, Brasil	17/03/2007	AP Fortuna-Perez/194	s/i
Nissolia schottii (Torr.) A. Gray	Sonora, México	24/02/1966	HD Ripley/14295	s/i
Nissolia tomentosa (Gardner) T.M. Moura & Fort.-Perez	São José do Barreiro, São Paulo, Brasil	02/05/1926	FC Hoehne/ n/a	NY
Poiretia angustifolia Vogel.	Luziânia, GO, Brasil	27/10/1990	Melo e França/381	CEN
Poiretia bahiana Cl. Müll.	Jacobina, BA, Brasil	28/06/1983	Coradin *et al.*/6168	CEN
Poiretia coriifolia Vogel.	Belo Horizonte, MG, Brasil	01/1934	Sampaio/6630	R
Poiretia crenata Cl. Müll.	Parque Nacional das Emas, GO, Brasil	09/04/2011	Fortuna-Perez/1592	BOTU
Poiretia elegans Cl. Müll.	Nova Roma, GO, Brasil	01/03/2000	Silva *et al.*/4312	NY
Poiretia latifolia Vogel	Guarapuava, PR, Brasil	19/02/2018	Mendes *et al.*/304	BOTU
Poiretia longipes Harms.	Costa Rica, MS, Brasil	01/04/2004	Pott et *al.*/11613	CGMS
Poiretia marginata Cl. Müll.	Frutal, MG, Brasil	19/01/1949	Krapovickas *et al.*/33077	NY
Poiretia punctata (Wiild.) Desv.	Cabeceira grande, MG, Brasil	28/03/2002	Pereira-Silva *et al.*/6380	CEN
Poiretia tetraphylla (Poir.) Burkart.	Gurinhatã, MG, Brasil	12/02/2013	Faria *et al.*/3267	CEN
Zornia brasiliensis Vogel	Tucano, BA, Brasil	26/08/2007	Fortuna-Perez *et al.*/295	BOTU
Zornia capensis Pers.	Quênia	14/12/1989	Robertson/6096	K

Zornia cearensis Huber	Campo Maior, PI, Brasil	12/06/1995	Nascimento & Alencar/1039	K
Zornia confusa Vanni	Diamantina, MG, Brasil	27/10/2006	Fortuna-Perez / n/a	BOTU
Zornia crinita (Mohlenbr.) Vanni	Cristalina, GO, Brasil	30/01/1990	Arbo *et al*./3153	K
Zornia cryptantha Arechav.	São Sebastião do Paraíso, MG, Brasil	20/11/2008	Fortuna-Perez *et al*./457	BOTU
Zornia curvata Mohlenbr.	Mucugê, BA, Brasil	14/03/2007	Fortuna-Perez *et al*./154	BOTU
Zornia dyctiocarpa DC.	Austrália	03/01/1994	n/a	K
Zornia echinocarpa (Moric. ex Meisn.) Benth.	Inhambupe, BA, Brasil	27/08/2007	Fortuna-Perez *et al*./305	BOTU
Zornia flemmingioides Moric.	Mucugê, BA, Brasil	12/03/2007	Fortuna-Perez *et al*./140	BOTU
Zornia gardneriana Moric.	Serra Branca, PI, Brasil	01/1907	Ule/7185	K
Zornia gibbosa Span.	Ilha de la reunión, África	08/04/1967	n/a	K
Zornia laevis Cham. & Schltdl.	México, Sonora	18/08/1935	n/a	K
Zornia latifolia Sm.	Campinas, SP, Brasil	25/3/2004	Fortuna-Perez/66	UEC
Zornia leptophylla (Benth.) Pittier	Iaçu, BA, Brasil	10/04/1992	Hatschbach *et al*./56976	K
Zornia milneana Mohlenbr.	Zambia, África	28/01/1952	Plowes/1410	K
Zornia mitziana Sousa Costa	Cristalina, GO, Brasil	14/08/2007	Fortuna-Perez *et al*./216	BOTU
Zornia myriadena Benth.	Palmeiras, BA, Brasil	15/03/2007	Fortuna-Perez *et al*./176	BOTU
Zornia pardina Mohlenbr.	Botucatu, SP, Brasil	11/03/1986	Bicudo/733	BOTU
Zornia piurensis Mohlenbr.	Peru, Piura	02/04/1939	Stork/11375	K
Zornia reptans Harms	Tanzânia, África	1898	Goetze/714	K
Zornia reticulata Sm.	Mucugê, BA, Brasil.	12/03/2007	Fortuna-Perez *et al*./140	BOTU
Zornia sericea Moric.	Inhambupé, BA, Brasil	27/8/2007	Fortuna-Perez *et al*./316	UEC
Zornia setosa Baker f.	Arusha, Tanzânia, África	16/01/1969	n/a	K
Zornia subsessilis Fort.-Perez & A.M.G. Azevedo	Joaquim Felício, MG, Brasil	11/2006	Fortuna-Perez *et al*./107	UEC
Zornia tenuifolia Moric.	Gentio do Ouro, BA, Brasil	24/08/2007	Fortuna-Perez *et al*./275	BOTU
Zornia venosa Mohlenbr.	Oaxaca, México	18/11/2001	n/a	K
Zornia virgata Moric.	Serra do Cipó, MG, Brasil	10/4/2008	Fortuna-Perez *et al*./445	BOTU

Table 2. Species with homobaric and heterobaric leaves of the Adesmia clade (Leguminosae-Papilionoideae), morphological characteristics of the bundle sheath extensions (BSE) and information on taxon habit and distribution.

Taxon	Leaf type according to the presence/absence of BSE	Vein order with BSE	Cellular composition of the BSE	Habit	Geographic distribution	Occurrence environments
Adesmia araujoi Burkart	Homobaric	-	-	Subshrub	Brazil endemic	Atlantic forest, Pampa
Adesmia arillata Miotto	Homobaric	-	-	Subshrub	Brazil endemic	Atlantic forest
Adesmia bicolor (Poir.) DC.	Homobaric	-	-	Subshrub	Argentina, Brazil, Chile, Uruguay	Pampa*
Adesmia ciliata Vogel	Homobaric	-	-	Subshrub	Brazil endemic	Atlantic forest, Pampa
Adesmia incana Volgel	Homobaric	-	-	Subshrub	Argentina, Brazil, Uruguay	Atlantic forest*, Pampa*
Adesmia latifolia (Spreng.) Vogel	Homobaric	-	-	Subshrub	Argentina, Brazil, Uruguay	Atlantic forest*, Pampa*
Adesmia muricata var *muricata* Gillies ex Hook. & Arn.	Homobaric	-	-	Subshrub	Brazil, Chile	Pampa*
Adesmia punctata var *hilariana* Benth.	Homobaric	-	-	Subshrub	Argentina, Brazil, Uruguay	Atlantic forest*, Pampa*
Adesmia punctata var *punctata* (Poir.) DC.	Homobaric	-	-	Subshrub	Argentina, Brazil, Uruguay	Pampa*
Adesmia reitziana Burkart	Homobaric	-	-	Subshrub	Brazil endemic	Atlantic forest
Adesmia rocinhensis Burkart	Homobaric	-	-	Subshrub	Brazil endemic	Atlantic forest
Adesmia securigerifolia Vogel	Homobaric	-	-	Subshrub	Argentina, Brazil, Uruguay	Pampa*
Adesmia tristis Volgel	Homobaric	-	-	Subshrub	Brazil endemic	Atlantic forest, Pampa
Amicia andicola (Griseb.) Harms	Heterobaric	2	Parenchyma	Subshrub	Argentina, Bolivia	Andes
Amicia fimbriata Harms ex Kuntze	Heterobaric	2	Parenchyma	Subshrub	Argentina, Bolivia	Andes
Amicia lobbiana Benth. ex Rusby	Heterobaric	2,3	Parenchyma / Sclerenchyma	Subshrub	Bolivia, Peru	Andes

Amicia medicaginea Griseb.	Heterobaric	2	Parenchyma	Subshrub	Argentina, Bolivia	Andes
Nissolia acutifolia (Vogel) T.M. Moura & Fort.-Perez	Heterobaric	2	Parenchyma	Vine	Brazil endemic	Atlantic forest
Nissolia blanchetiana (Benth.) T.M. Moura & Fort.-Perez	Heterobaric	2	Parenchyma	Vine	Brazil endemic	Caatinga, Brazilian Cerrado, Atlantic Forest
Nissolia bracteosa (Rudd) T.M. Moura & Fort.-Perez	Heterobaric	2,3	Parenchyma	Vine	Brazil endemic	Caatinga, Brazilian Cerrado
Nissolia brasiliensis (Vogel) T.M. Moura & Fort.-Perez	Heterobaric	2,3	Parenchyma	Vine	Mexico, Argentina, Belize, Bolivia, Brazil, Colombia, Ecuador, Guatemala, Mexico, Paraguay, Peru, Suriname, Trinidad	Amazon Forest*, Caatinga*, Brazilian Cerrado, Atlantic Forest*
Nissolia chacoensis (Vanni) T.M. Moura & Fort.-Perez	Heterobaric	2,3	Parenchyma	Vine	Bolivia, Paraguay	Amazon Forest
Nissolia chiapensis Rudd.	Heterobaric	2,3	Parenchyma	Vine	Mexico, Guatemala	Tropical Forest
Nissolia fruticosa Jacq.	Heterobaric	2,3	Parenchyma	Vine	Brazil, Colombia, Costa Rica, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Venezuela	Amazon Forest*, Pantanal*
Nissolia gentryi Rudd.	Heterobaric	2	Parenchyma	Vine	Mexico endermic	Desert
Nissolia klugii (Rudd) T.M. Moura & Fort.-Perez	Heterobaric	2	Parenchyma	Vine	Brazil, Ecuador, Peru	Amazon Forest *
Nissolia latisiliqua (Poir.) T.M. Moura & Fort.-Perez	Heterobaric	2,3	Parenchyma	Vine	Colombia, Costa Rica, Ecuador, Panama	Desert
Nissolia laxior (B.L. Rob.) Rose	Heterobaric	2,3	Parenchyma	Vine	Mexico endemic	Desert
Nissolia leiogyne Sandwith	Heterobaric	2,3	Parenchyma	Vine	Mexico endemic	Semi-arid

Nissolia longiflora (Benth. ex A. Gray) T.M. Moura & Fort.-Perez	Heterobaric	2,3	Parenchyma	Vine	Brazil, Bolívia	Amazon Forest, Caatinga*, Brazilian Cerrado*, Atlantic Forest*
Nissolia microptera Poir.	Heterobaric	2,3	Parenchyma	Vine	Mexico endemic	n/a
Nissolia nigricans (Burkart) T.M. Moura & Fort.-Perez	Heterobaric	2,3	Parenchyma	Vine	Brazil, Argentina, Uruguay	Atlantic Forest *
Nissolia platycalyx S. Watson	Heterobaric	2,3	Parenchyma	Vine	USA, México	Semi-arid/Desert
Nissolia platycarpa Benth.	Heterobaric	2,3	Parenchyma	Vine	Mexico endemic	Deciduous forest
Nissolia vincentina (Ker Gawl.) T.M. Moura & Fort.-Perez	Heterobaric	2,3	Parenchyma	Vine	Antilles, Brazil, Colombia, Dominican Republic, Mexico, Venezuela	Amazon Forest, Caatinga*, Brazilian Cerrado*, Atlantic Forest*
Nissolia schottii (Torr.) A. Gray	Heterobaric	2,3	Parenchyma	Vine	USA, México	Desert
Nissolia tomentosa (Gardner) T.M. Moura & Fort.-Perez	Heterobaric	2,3	Parenchyma	Vine	Brazil endemic	Atlantic Forest*
Nissolia wislizeni (A. Gray) A. Gray	Heterobaric	2,3	Parenchyma	Subshrub	USA, México	Desert
Poiretia angustifolia Vogel.	Heterobaric	2	Parenchyma	Subshrub	Brazil endemic	Brazilian Cerrado, Atlantic Forest
Poiretia bahiana Cl. Müll.	Heterobaric	2,3	Parenchyma	Subshrub	Brazil endemic	Brazilian Cerrado, Caatinga
Poiretia coriifolia Vogel.	Heterobaric	2,3,4	Sclerenchyma / Parenchyma	Subshrub	Brazil endemic	Brazilian Cerrado
Poiretia crenata Cl. Müll.	Heterobaric	2	Parenchyma	Subshrub	Brazil endemic	Brazilian Cerrado
Poiretia elegans Cl. Müll.	Heterobaric	2	Parenchyma	Shrub	Brazil endemic	Brazilian Cerrado
Poiretia latifolia Vogel	Heterobaric	2,3,4	Sclerenchyma / Parenchyma	Subshrub	Argentina, Brazil, Paraguay e Uruguay	Brazilian Cerrado*, Atlantic Forest*, Pampa
Poiretia longipes Harms.	Homobaric	-	-	Subshrub	Brazil endemic	Brazilian Cerrado
Poiretia marginata Cl. Müll.	Heterobaric	2,3	Sclerenchyma / Parenchyma	Subshrub	Brazil endemic	Brazilian Cerrado

Poiretia punctata (Wiild.) Desv.	Heterobaric	2,3	Parenchyma	Vine	Central and South America	Caatinga*, Brazilian Cerrado*, Atlantic Forest*
Poiretia tetraphylla (Poir.) Burkart.	Heterobaric	2	Parenchyma	Subshrub	Brazil, Paraguay, Uruguay	Brazilian Cerrado*, Atlantic Forest
Zornia brasiliensis Vogel	Homobaric	-	-	Subshrub	Brazil, Venezuela	Brazilian Cerrado*, Caatinga*, Atlantic Forest*, Amazon Forest
Zornia cearensis Huber	Homobaric	-	-	Subshrub	Brazil endemic	Brazilian Cerrado, Caatinga, Atlantic Forest
Zornia confusa Vanni	Homobaric	-	-	Subshrub	Brazil, Costa Rica, Guatemala, Honduras, Panamá, Mexico, Venezuela	Brazilian Cerrado*, Atlantic Forest*
Zornia crinita (Mohlenbr.) Vanni	Heterobaric	2	Parenchyma	Subshrub	Argentina, Bolivia, Brazil, French Guiana, Venezuela, Paraguay, Suriname	Brazilian Cerrado*, Pampa, Pantanal*
Zornia cryptantha Arechav.	Homobaric	-	-	Subshrub	Argentina, Bolivia, Colombia, Paraguay, Uruguay, Venezuela Brazil	Brazilian Cerrado*, Atlantic Forest*, Pampa
Zornia curvata Mohlenbr.	Homobaric	-	-	Subshrub	North, central and South America	Amazon Forest*, Caatinga*, Brazilian Cerrado*, Atlantic Forest*, Pantanal*, Pampa
Zornia dyctiocarpa DC.	Homobaric	-	-	Subshrub	Australia	
Zornia echinocarpa (Moric. ex Meisn.) Benth.	Homobaric	-	-	Subshrub	Brazil endemic	Caatinga
Zornia flemmingioides Moric.	Homobaric	-	-	Subshrub	Brazil endemic	Brazilian Cerrado, Caatinga
Zornia gardneriana Moric.	Homobaric	-	-	Subshrub	Brazil endemic	Caatinga

Zornia gibbosa Span.	Heterobaric	2	Parenchyma	Subshrub	Asia	Wide distribution (anthropogenic environments) across Asia
Zornia laevis Cham. & Schltdl.	Homobaric	-	-	Subshrub	North, central and South America, Australia, Asia	n/a
Zornia latifolia Sm.	Heterobaric	2	Parenchyma	Subshrub	Argentina, Bolivia, Brazil, Ecuador, Paraguay, Uruguay, Venezuela; Central America, Africa	Amazon Forest*, Caatinga*, Brazilian Cerrado*, Atlantic Forest*, Pampa*, Pantanal *
Zornia leptophylla (Benth.) Pittier	Homobaric	-	-	Subshrub	Brazil e Colombia	Amazon Forest*, Caatinga*, Brazilian Cerrado*
Zornia milneana Mohlenbr.	Homobaric	-	-	Subshrub	Africa	Anthropogenic Landscapes, Prairies and Forests of Zambezia
Zornia mitziana Sousa Costa	Heterobaric	2	Parenchyma	Subshrub	Brazil endemic	Brazilian Cerrado
Zornia myriadena Benth.	Homobaric	-	-	Subshrub	Cuba, Jamaica, Brazil	Brazilian Cerrado*, Caatinga*
Zornia pardina Mohlenbr.	Heterobaric	2,3	Parenchyma	Subshrub	Argentina, Brazil, French Guiana, Paraguay, Uruguay, Venezuela	Brazilian Cerrado*, Atlantic Forest*
Zornia piurensis Mohlenbr.	Homobaric	-	-	Subshrub	Peru	Drought regions on slopes
Zornia reptans Harms	Heterobaric	2	Parenchyma	Subshrub	Tanzania	Zambezia Forest
Zornia reticulata Sm.	Homobaric	-	-	Subshrub	Central and South America	Amazon Forest*, Caatinga*, Brazilian Cerrado*, Atlantic Forest*, Pampa*, Pantanal*

Zornia sericea Moric.	Homobaric	-	Subshrub	Brazil, Paraguay, Bolivia, Venezuela	Amazon Forest*, Caatinga*, Brazilian Cerrado*
Zornia setosa Baker f.	Homobaric	-	Subshrub	Africa	Tropical Savanna
Zornia subsessilis Fort.-Perez & A.M.G. Azevedo	Homobaric	-	Subshrub	Brazil endemic	Brazilian Cerrado
Zornia tenuifolia Moric.	Homobaric	-	Subshrub	Brazil endemic	Caatinga
Zornia venosa Mohlenbr.	Homobaric	-	Subshrub	Mexico	Grassy open fields
Zornia virgata Moric.	Homobaric	-	Subshrub	Brazil endemic	Brazilian Cerrado, Atlantic Forest

- Phytogeographic domains with an asterisk (*) correspond only to the regions of Brazil.

Considerações finais

Este trabalho foi dedicado ao estudo das extensões de bainha do feixe (EBF) em folhas de leguminosas, com foco em seus aspectos ultraestruturais e fisiológicos, buscando compreender possíveis funções das EBF para o corpo vegetal, além de investigar os aspectos evolutivos dessa caraterística anatômica em espécies de um determinado clado.

O desenvolvimento de um estudo multidisciplinar foi essencial para uma visão mais ampla das EBF. A microscopia de transmissão permitiu observar as características subcelulares das células das bainhas dos feixes em folhas homobáricas e heterobáricas, mostrando a existência de peculiaridades relacionadas ao funcionamento dessas folhas. Diferenças entre os tipos foliares foram encontradas principalmente quanto ao desenvolvimento de estruturas de comunicação simplástica e dos plastídios. De acordo com o nosso conhecimento, esse foi o primeiro estudo a comparar a ultraestrutura das células da bainha dos feixes em folhas homobáricas e heterobáricas. A análise de um maior número de espécies ao microscópio eletrônico de transmissão só não foi possível devido a problemas técnicos com os equipamentos do Centro de Microscopia Eletrônica do IBB, UNESP.

A realização de um estudo experimental foi fundamental para o entendimento das respostas morfofuncionais de espécies de leguminosas nativas do cerrado, portadoras de diferentes tipos foliares, a variações na intensidade luminosa e disponibilidade hídrica. Embora não tenhamos registrado diferenças no desenvolvimento das bainhas dos feixes entre os diferentes tipos foliares, nossos dados fisiológicos também apontaram que a espécie com folhas heterobáricas apresentou maior eficiência com relação ao uso da água, mesmo em condições de alta luminosidade e restrição hídrica. Esses dados apontam para diferentes estratégias de espécies com folhas homobáricas e heterobáricas para lidar com fatores ambientais. Nossos resultados mostram ainda que espécies com diferentes tipos foliares do cerrado parecem responder a variações na luz e disponibilidade hídrica de maneira distinta de espécies de clima temperado.

A análise da distribuição de espécies com folhas homobáricas e heterobáricas em um determinado grupo também é uma abordagem inédita. O estudo anatômico das folhas de 75 espécies de leguminosas pertencentes ao clado Adesmia nos permitiu inferir que a presença das EBF parece estar relacionada ao hábito de vida das plantas, o que consequentemente pode ter ligação com fatores microambientais, tais como luminosidade e disponibilidade de hídrica. Além disso, a presença ou ausência das EBF parece estar relacionada aos grupos taxonômicos. Dentro do clado Adesmia, essa característica parece ter evoluído de folhas heterobáricas com extensões de bainha em nervuras de segunda, terceira e quarta ordem para folhas com EBF apenas nas nervuras de segunda ordem e, nas linhagens mais recentes, para folhas homobáricas.

Os dados produzidos abrem novas perspectivas para o estudo do desenvolvimento e funcionamento das EBF. Pesquisas adicionais de cunho experimental sobre a influência de fatores ambientais no desenvolvimento e funcionamento de folhas homobáricas e heterobáricas envolvendo um maior número de espécies devem ser realizadas para confirmar se as respostas observadas nesse estudo são comuns às espécies de clima tropical. Ainda, análises ultraestruturais de um maior número de espécies com folhas homobáricas e heterobáricas devem ser realizadas para que possamos generalizar as peculiaridades subcelulares aqui reportadas para os diferentes tipos foliares.

Por fim, os dados obtidos geram informações bastante importantes e inovadoras sobre os aspectos estruturais, ecológicos, fisiológicos e evolutivos das EBF e abrem novas perspectivas para pesquisas mais aprofundadas nesse tema, especialmente envolvendo plantas do cerrado brasileiro.

Sumário