FIBRAS GELATINOSAS EM *Eriosema* (DC.) Desv. (LEGUMINOSAE): DISTRIBUIÇÃO E CARACTERIZAÇÃO ESTRUTURAL E QUÍMICA

TAYEME CRISTINA PIVA

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

BOTUCATU – SP
2020

UNIVERSIDADE ESTADUAL PAULISTA
"Júlio de Mesquita Filho"

INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

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FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP

BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE-CRB 8/5651

Piva, Tayeme Cristina.
Fibras gelatinosas em *Eriosema* (DC.) Desv. (Leguminosae): distribuição e caracterização estrutural e química / Tayeme Cristina Piva. - Botucatu, 2020

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu
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Capes: 20302037

1. Leguminosa. 2. Ecologia dos cerrados. 3. Parede celular vegetal.

Palavras-chave: Camada-G; Camada-G composta; Cerrado; Fibras gelatinosas; Parede celular.

“Dedico aos meus amados e
queridos pais
Luiz Alberto e Kátia”

AGRADECIMENTOS

Foram muitos os que contribuíram para a geração e finalização deste trabalho, mais um marco importante da minha vida profissional, cabe aqui meus mais sinceros agradecimentos.

Primeiramente gostaria de agradecer a CAPES pela concessão da bolsa de estudos e a Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências e Departamento de Botânica por todo apoio financeiro necessário para esta pesquisa.

Gostaria de agradecer a Profª Silvia Rodrigues Machado por me incentivar sempre buscar em mim a melhor profissional que eu possa ser, por ser uma pessoa inspiradora e iluminada. Gostaria de agradecer também por toda sua paciência, apoio e confiança durante esses quatro anos. Nunca se esqueça sou sua fã número 1 (eu tenho ciúmes hahaha) e sou muito grata por poder conviver com a senhora todo esse tempo, eu quero ser pelo menos um terço para meus alunos o que a senhora é para mim. Muita admiração e gratidão pela senhora!

À Profª Ana Paula por me mostrar o universo das Eriosemas, por todo apoio, por todo material coletado e por todos os ensinamentos, muito obrigada!

Á Profª Edna Scremin Dias por me dar a oportunidade de conhecer e aflorar em mim todo esse amor e curiosidade que tenho pelas fibras gelatinosas. Muito obrigada!

À Profª Carmen Marcati por sempre me ajudar independentemente da situação, seja em seu laboratório ou no hospital, a senhora é um exemplo a ser seguido. Quero um dia ter pelo menos a metade desse brilho nos olhos como a senhora tem quando ensina.

À todos os professores do Programa de Pós-Graduação em Botânica, pelos ensinamentos passados, apoio e dedicação. Muito obrigada!

À Profª Tatyana Gorshkova, Olya, Alsu, Marina, Natasha Mokshina, Natasha Gogoleva, Polina, Ayruz, Ludmilla, Ilzira e Anna por me ajudarem tanto na minha estadia na Rússia, por toda paciência em me ensinar e por permitir que a melhor experiência da minha vida fosse tão leve, enriquecedora e produtiva.

Aos técnicos da Botânica, do Centro de Microscopia Eletrônica e do Laboratório de Anatomia da Madeira por todo auxilio.

À Deus por toda força e graça, por me permitir viver e usufruir a vida da melhor forma possível, por sempre estar comigo em todos os momentos, nas alegrias e nas tristezas, ao longo de toda minha vida.

Aos meus queridos pais, Bigode e Katinha, minha eterna gratidão, por todo apoio dado desde o começo da minha vida acadêmica, sempre me incentivando mesmo que de longe, se fazendo presente com um telefonema, uma mensagem ou um pensamento. Esse apoio de vocês foi essencial e primordial para que eu alcançasse todos os meus sonhos. Vocês

dois são a minha razão de viver e os amores da minha vida! Sem vocês, eu não sou nada! Amo vocês!

Ao meu irmão Gustavo e meus sobrinhos Felipe e Arthur por todo carinho, apoio, incentivo e principalmente por todas as risadas que vocês, os homens da minha vida, me proporcionaram, vocês são meus amores!

Aos meus familiares: tios, tias, primos e primas, seja pelo laço de sangue ou pelo coração, por toda torcida ao longo da minha jornada profissional, por sempre enviarem energias positivas para mim, independentemente de onde eu estivesse.

Gostaria de agradecer imensamente a minha família Botucuda! Sem vocês aqui, minha jornada em Botucatu não teria sido a mesmo, sei que aqui fiz além de amigos, fiz uma família. Principalmente às minhas lindezas: Angélica (Angel), Bruna (Cine), Camila (Rã), Diana (Didi), Fernanda (Ferzinha), Janaína (Jana), Juliana (July), Katiane (Kat), Loraine (Lo), Lorena (Lolo), Luísa (Luli), Marília (Suin), Nathália (Nath), Thais (Tata), Vanessa (Van) e Wanderléia (Wandinha); migas sem vocês, minhas companheiras, eu não teria vivido esses quatro anos tão maravilhosamente bem... vocês são para toda vida!

Aos meus amigos do LAPAV e departamento, por todo cafezinho/chazinho, por toda troca de conhecimento, apoio e incentivo seja na pesquisa ou fora dela.

Aos meus amigos e minhas amigas que fiz enquanto morava em Araraquara, Bandeirantes e Campo Grande por toda palavra de carinho e apoio sempre, mesmo que de longe.

À Gloria, Simone e Katielle por cuidarem com tanto carrinho de mim todo esse tempo!

À todas as pessoas que colaboraram para a realização deste trabalho, diretamente ou indiretamente, meus sinceros agradecimentos!

SUMÁRIO

Este trabalho será apresentado em dois capítulos, o primeiro intitulado "Variations in the architecture and histochemistry of the gelatinous fibers of *Eriosema* (DC.) Desv. (Leguminosae) species from Brazilian Cerrado" e o segundo "Detection *in situ* of lignin and immunohistochemical study in *Eriosema simplicifolium* (Kunth) G.Don. (Leguminosae): A suitable approach for improve our understanding on gelatinous fibers constitution across the vegetative body"

RESUMO GERAL

Fibras gelatinosas são células especializadas de esclerênquima que possuem uma camada interna altamente modificada de aparência gelatinosa e translúcida, denominada camada-G. Diversas funções são atribuídas as fibras gelatinosas sendo a principal e mais conhecida a sua relação com o lenho de tração. Porém, outras funções de cunho fisiológico e adaptativo, tais como armazenamento de água e manutenção da orientação foliar foram descritas para as fibras gelatinosas. As fibras gelatinosas são reportadas em diversas famílias de angiospermas nos mais diversos ambientes, com isso outras funções podem ser associadas a ocorrência das fibras gelatinosas nos mais diferentes ambientes. O objetivo desse estudo foi analisar as fibras gelatinosas em representantes de *Eriosema* (DC.) Desv. um gênero de leguminosa comumente encontrado em Cerrado, visando a acessar variações na distribuição e organização das fibras gelatinosas no eixo vegetativo aéreo e subterrâneo, caracterizar a estrutura básica e a histoquímica das paredes celulares. O estudo foi conduzido com 19 táxons coletados em diferentes áreas de Cerrado, sendo amostrados a folha, caule aéreo e órgão subterrâneo. O material foi processado de acordo com técnicas usuais em anatomia e histoquímica vegetal. Uma espécie foi considerada como modelo e foram utilizados os anticorpos LM5 e LM10 marcadores para galactanos e xilanos, respectivamente, nas paredes das fibras gelatinosas. As fibras gelatinosas ocorreram por todo o corpo vegetativo dos táxons de *Eriosema* estudados, sendo extraxilemáticas em regiões com crescimento primário e xilemáticas em regiões com crescimento secundário. Quanto à organização, na folha as fibras gelatinosas formaram calotas ao redor do floema primário da nervura principal; no caule, se organizaram em cordão cilíndrico completo ao redor do floema em crescimento primário ou em pequenos grupos separados por parênquima em crescimento secundário; no órgão subterrâneo, se organizaram em faixas tangenciais separadas por raios de parênquima xilemático. Em todos os órgãos analisados, as fibras gelatinosas exibiram três camadas parietais distintas: camada-P, incluindo a parede primária e lamela média rica em pectina; a camada-S com número variável de estratos (1-3) geralmente lignificados, e a camada-G, parede celular terciária, não lignificada ou com baixo teor de lignina. De um modo geral, a camada-G foi mais espessa que as demais camadas parietais (P e S). A espécie modelo, apresentou camada-G composta (G+L) formada por lamela não-lignificada (G) e lamela interna lignificada (L) ocorreu na folha (região da nervura principal), regiões do caule em crescimento secundário e órgão subterrâneo. A presença de lignina e xilanos na lamela

interna lignificada (L) da camada-G composta foi demonstrada neste estudo, por meio de métodos histoquímico e imunohistoquimico, respectivamente. A ausência de lignina na camada-G, ao conferir propriedades hidrofílicas a esta camada parietal, pode favorecer o armazenamento de água a curto período. Porém, a presença de lignina na camada-G composta (camada-L), como observado no eixo vegetativo aéreo e subterrâneo, ao conferir rigidez e resistência mecânica permitiria proteção em condições ambientais adversas e maior resistência ao fogo. Considerando a abundância de fibras gelatinosas no eixo vegetativo dos táxons de *Eriosema* aqui analisados, tais fibras podem representar uma adaptação estrutural associada às condições do Cerrado, um ambiente sazonal com temperaturas elevadas e alta incidência luminosa, períodos alternados de alta e baixa disponibilidade de água, solos pobres em nutrientes e aluminotóxicos e queimadas recorrentes. Estudos experimentais com foco na ontogênese da camada-G composta nas fibras gelatinosas de plantas sujeitas a diferentes condições edáficas e climáticas serão necessários para um melhor entendimento sobre os fatores ambientais que favorecem a sua formação.

Palavras-chave: Camada-G, Camada-G composta, Cerrado, Fibras gelatinosas, Parede celular

ABSTRACT

Gelatinous fibers (G-fibers) are specialized sclerenchyma cells that have a highly modified, translucent, gelatinous appearance inner layer called the G-layer. Several functions are attributed to the G-fibers being the main and best known its relation with the tension wood. However, other physiological and adaptive functions, such as water storage and maintenance of leaf orientation, have been described for G-fibers. G-fibers are reported in several angiosperm families in the most diverse environments, so other functions may be associated with the occurrence of G-fibers in the most different environments. The purpose of this study was to analyze the G-fibers in representatives of *Eriosema* (DC.) Desv. a genus of legumes commonly found in Cerrado, aiming to access variations in the distribution and organization of G-fibers in the aerial and underground vegetative axis, to characterize the basic structure and histochemistry of cell walls. The study was conducted with 19 taxa collected in different areas of Cerrado, being sampled the leaf, aerial stem and underground organ. The material was processed according to usual techniques in plant anatomy and histochemistry. LM5 and LM10 antibodies that mark galactans and xylans, respectively, were used on the G-fibers walls. G-fibers occurred throughout the vegetative body of the studied *Eriosema* taxa, being extraxylary in regions with primary growth and xylary in regions with secondary growth. As for the organization, in the leaf the G-fibers formed caps along the primary phloem of the midrib; in the stem, they were organized in complete cylindrical strands around the primary growing phloem or in small groups separated by secondary growing parenchyma; in the underground organ, organized into tangential bands separated by xylem parenchyma rays. In all organs analyzed, the G-fibers exhibited three distinct parietal layers: P-layer, including the primary wall and pectin-rich middle lamella; the S-layer with variable number of strata (1-3) generally lignified, and the G-layer, non-lignified or low lignin tertiary cell wall. In general, the G-layer has always been thicker than the other parietal layers (P and S). Composite G-layer (G+L) formed by non-lignified lamella (G) and lignified internal lamella (L) occurred in the leaf (midrib region), stem regions in secondary growth and underground organ. The presence of lignin and xylans in the lignified internal lamellae (L) of the composed G-layer was demonstrated in this study by histochemical and immunohistochemical methods, respectively.

The absence of lignin in the G-layer, by conferring hydrophilic properties to this parietal layer, may favor short-term water storage. However, the presence of lignin in the composed G-layer (L-layer), as observed in the aerial and underground vegetative axis, by conferring rigidity and mechanical strength would allow protection in adverse environmental conditions and greater fire resistance. Considering the abundance of G-fibers in the vegetative axis of the *Eriosema* taxa analyzed here, such fibers may represent a structural adaptation associated with Cerrado conditions, a seasonal environment with high temperatures and high light incidence, alternating periods of high and low water availability, nutrient-poor and aluminotoxic soils and recurrent burning. Experimental studies focusing on the composite G-layer ontogenesis in G-fibers of plants subjected to different edaphic and climatic conditions will be necessary for a better understanding of the environmental factors that favor their formation.

Keywords: Cerrado, cell wall, composed G-layer, G-fibers and G-layer.

INTRODUÇÃO GERAL

Fibras gelatinosas, um tipo especializado de célula do esclerênquima, são assim chamadas devido à presença de uma camada parietal interna altamente modificada, de aparência gelatinosa e translúcida, localizada mais internamente na parede celular secundária, distinguindo-se das demais camadas (Metcalfe & Chalk 1950; Dickison, 2000; Clair et al. 2008; Scatena & Scremin-Dias 2012; Evert 2013; Pace & Soffiatti 2013; Ruelle 2014). Esta camada parietal peculiar, comparada a favo de mel quando intumescida (Sachsse 1962, 1964, 1965), foi descrita pela primeira vez em 1860 por Theodor Hartig, sendo denominada "camada mucilaginosa", "camada cartilaginosa" ou "camada gelatinosa" devido ao seu conteúdo de aspecto gelatinoso (Ruelle 2014). Em 1964, o comitê da Associação Internacional de Anatomia de Madeira nomeou essa camada de "Camada-G" no "Glossário Multilíngue de termos usados em madeira" (IAWA-Committee 1964).

A camada-G se caracteriza pela aparência semelhante a uma gelatina com menor ângulo de microfibrila (Washusen & Evans 2001; Ruelle et al. 2007), alta mesoposidade (Chang et al. 2009), alto teor de celulose cristalina (Chernova & Gorshkova 2007), baixo teor de lignina (Fahn 1990; Evert 2006; Mellerowicz & Gorshkova 2012) e alta concentração de galactanos (Gorshkova & Morvan 2006). A camada-G se contrai durante o dessecamento, frequentemente resultando em fibras com aspecto convoluto (Dickison 2000; Evert 2006).

Ultraestruturalmente, fibras gelatinosas maduras são caracterizadas pela presença de três camadas parietais distintas: camada-P, que compreende a parede primária incluindo a região mais externa da lamela média rica em pectina; a camada-S, com número variável de estratos (1-3) geralmente lignificados, e a camada-G, terciária, mesoporosa, não lignificada ou com baixo teor de lignina (Wardrop & Dadswell 1948; Ruelle et al. 2007; Chang et al. 2009).

Estudos sobre o desenvolvimento de fibras gelatinosas relataram que a camada-G composta que pode exibir multicamadas, ou seja, apresenta subcamadas distintas (Gorshkova et al. 2004, 2010; Ghislain et al. 2016). Tanto a espessura quanto o aspecto dessas duas camadas se modificam durante a formação da camada-G, indicando que a

parede das fibras gelatinosas é remodelada durante o desenvolvimento (Gorshkova et al. 2004, 2010).

Estudos recentes têm demonstrado que as camadas P, S e G das fibras gelatinosas diferem substancialmente entre si. De modo geral, os conjuntos de polímeros nas camadas P e S são semelhantes e representam, respectivamente, a composição geral das paredes celulares primárias e secundárias de muitas células vegetais. Em contraste, a camada-G é muito distinta das demais camadas de parede celular em três aspectos principais: i) orientação, conteúdo e estrutura das fibrilas de celulose; ii) conjunto de polissacarídeos da matriz e iii) conteúdo de lignina (Mellerowicz & Gorshkova 2012).

A arquitetura das paredes celulares das fibras gelatinosas e das fibras de esclerênquima típicas parece representar dois extremos do mesmo contínuo - a primeira com baixos teores ou ausência de lignina e baixo ângulo de microfibrilas de celulose, e a segunda com altos teores de lignina e elevado ângulo de celulose (Mellerowicz & Gorshkova 2012). Estudos têm demonstrado que existem diferenças nos tipos de polímero da matriz e sua organização que resultam em diferenças significativas na arquitetura da parede celular das fibras gelatinosas (Snegireva et al. 2010; Gorshkova et al. 2012; Mellerowicz & Gorshkova 2012).

De um modo geral, a composição da camada-G contrasta fortemente com a composição das camadas S das fibras gelatinosas, que contêm cerca de 50% de celulose, 30% de hemiceluloses (incluindo 25% xilano e 5% de glucomanano) e 20% de lignina (Timell 1967; Mellerowicz et al. 2001; Awano et al. 2002). Embora a lignina seja um componente comum das camadas S das fibras gelatinosas, a ausência total de lignina na camada-G tem sido reportada em várias espécies (Love et al. 1994; Gorshkova et al. 2000; Plomion et al. 2001; Pilate et al. 2004; Meloche et al. 2007; Bond et al. 2008; Kaku et al. 2009; Schreiber et al. 2010). No entanto, existem vários relatos sobre a presença de alguns compostos aromáticos por toda a extensão da camada-G (Joseleau et al. 2004; Lehringer et al. 2008) ou em sua porção mais interna (Gierlinger & Schwanninger 2006). Assim, a presença/ausência de lignina na camada-G é controversa e questionável e constitui objeto de estudos.

Gorshkova et al. (2018) consideram a camada-G como uma camada interna adicional, constituindo a parede celular terciária uma vez que se desenvolve após a parede celular primária e secundária; possui composição, arquitetura e propriedades físicas

distintas das demais camadas e sua formação é regulada por um conjunto de fatores na transcrição celular que diferem dos fatores presentes nas paredes celulares primária e secundária. Para outros autores, a camada-G pode constituir uma parede adicional ou substituir uma ou mais camadas da parede celular secundária (Singh & Donaldson 1999; Donaldson & Turner 2001). Em função desta organização, três tipos de arranjo foram descritos nas paredes das fibras gelatinosas: P + S1 + S2 + S3 + G; P + S1 + S2 + G; P + S1 + G. No entanto, em espécies tropicais há relados de fibras gelatinosas com estrutura de camada-G composta, pode ser organizada em S1 + S2 + n (G + L), onde n é o número de repetições na camada-G não lignificada (G) e a camada fina lignificada (L) (Clair et al. 2006; Ruelle et al. 2007; Nakagawa et al. 2012; Nakagawa et al. 2014).

As fibras gelatinosas são tradicionalmente estudadas em lenho de tração, recebendo o nome de "fibras de reação", sendo sua presença interpretada como uma resposta ao estresse tensional dentro de órgãos desenvolvidos e permitindo a sua movimentação (Wardrop 1964; Yoshida et al. 2002; Clair et al. 2003; Fang et al. 2008; Abasolo et al. 2009); porém nem sempre é possível verificar a camada-G quando a madeira está sob tração (Clair et al. 2006). Entretanto, fibras gelatinosas têm sido encontradas também em órgãos que estão sujeitos a esta tração como em crescimento primário ou secundário, associadas ou não aos tecidos vasculares (Tomlinson 2003; Gorshkova & Morvan 2006; Toghraie et al. 2006) tais como gavinhas, espinhos, caules e pedúnculos (Mellerowicz & Gorshkova 2012), raízes normais ou contráteis (Fisher 2008; Schreiber et al. 2010; Tomlinson et al. 2014; Bonacorsi & Seago 2016; Magellan et al. 2018), órgãos subterrâneos espessados (Paviani 1974; Fisher 2008; Silva 2011; Yule 2012) e folhas (Barros et al. 2014; Graciano-Ribeiro et al. 2016; Guerra & Scremin-Dias 2018).

Fibras gelatinosas têm sido observadas em diversos táxons, incluindo gimnospermas (Jourez 1997; Lev-Yadun 1999; Tomlinson 2003), pteridófitas (Gierlinger et al. 2008); contudo, são encontradas principalmente em angiospermas lenhosas onde a abundância de fibras gelatinosas pode variar consideravelmente, mesmo em espécies relacionadas (Mellerowicz & Gorshkova 2012; Ghislain & Clair 2017). Apesar da ampla ocorrência de fibras gelatinosas em diferentes grupos de plantas, faltam estudos detalhados sobre a arquitetura de suas paredes e composição da camada gelatinosa nos diferentes táxons.

Em razão das características da camada-G, diversas funções têm sido atribuídas às fibras gelatinosas, além daquela tradicionalmente atribuída às fibras de reação que é gerar força tensional sob estresse (Mellerowicz & Gorshkova 2012 e literatura citada). Fibras gelatinosas podem armazenar água, sendo importantes na fisiologia das plantas de ambientes com temperaturas extremas, sofrendo déficit hídrico (Lyshede 1978; Paviani 1974, 1978; Marcati et al. 2001; Luchi 2004) e solos aluminotóxicos, atuando na compartimentalização deste elemento em plantas acumuladoras de alumínio (Milanez et al. 2017). As fibras gelatinosas também podem atuar na manutenção da posição ereta de caules em desenvolvimento (Fisher & Stevenson 1981), na orientação foliar em relação à gravidade e à posição do sol (Sperry 1982) e na proteção às gemas laterais e cotilédones em ambientes sujeitos ao fogo, na contração ou flexão do hipocótilo e/ou órgão subterrâneo (Fisher 2008). A função de contrção e flexão, é similar àquela descrita por Bell e Bryan (1991) para as raízes contráteis, que são capazes de contrações periódicas, permitindo o aprofundamento de bulbos, cormos e rizomas, quando em condições adversas ocasionando a proteção dos meristemas caulinares.

Recentemente as fibras gelatinosas têm sido alvo de estudos por diferentes razões. A composição química de suas paredes (baixo teor ou ausência de lignina, baixo pentosano e altos teores de celulose cristalina), facilita a decomposição dessas fibras por enzimas microbianas tornando-as apropriadas para uso como matéria prima para a produção de biocombustíveis (Engels & Jung 1998), além de fornecer matéria-prima para sacarificação (conversão do amido em açúcar industrial) (Mellorowicz & Gorshkova 2012), podendo levar um melhor rendimento de glicose (Brereton et al. 2011, 2012). Além disso, estudos que visam a compreender os mecanismos celulares e moleculares responsáveis pela geração de estresse tensional nessas fibras poderiam potencialmente conduzir ao desenvolvimento de novos materiais com propriedades similares (biomiméticos) (Mellerowicz & Gorshkova 2012).

A ampla ocorrência de fibras gelatinosas em diferentes grupos de plantas vasculares indica que essa especialização do esclerênquima surgiu em um estágio inicial da evolução das plantas terrestres (Mellerowicz & Gorshkova 2012) e sua ocorrência possibilitaria a sobrevivência das plantas em condições ambientais adversas (Böchek & Lyshede 1972; Schreiber et al. 2010). Contudo, a comprovação dos prováveis papéis atribuídos às fibras gelatinosas exige estudos detalhados em níveis inter e intraespecíficos, que comparem a sua distribuição e abundância, bem como, a estrutura e

a composição da parede celular nos diferentes órgãos da planta. Estudos com esta abordagem permitem acessar possíveis variações na distribuição e características estruturais e histoquímicas das paredes das fibras gelatinosas, e associar tais aspectos com os possíveis papéis desempenhados.

Alguns estudos reportam a ocorrência de fibras gelatinosas em espécies da família de Leguminosae em órgão subterrâneos (Fisher 2008; Schreiber et al. 2010; Silva 2011; Yule 2012), caules em crescimentos primários e secundários (Marcati et al. 2001; Piva et al. 2019) e folhas (Paviani 1974; Barros et al. 2014; Guerra e Scremin-Dias 2018; Seixas et al. 2019; Piva et al. 2019), porém poucos desses estudos trazem de forma detalhada informações sobre as fibras gelatinosas. Com base nisto, foi selecionado na família Leguminosae, o gênero *Eriosema* (DC.) Desv. para desenvolver um estudo mais detalhado sobre as fibras gelatinosas uma vez que já existia um conhecimento prévio destas fibras em algumas espécies (Piva et al., 2019).

Eriosema é um gênero atualmente incluído na tribo Phaseoleae, subtribo Cajaninae, Leguminosae (Schrire 2005; Cândido et al. 2014), e possui cerca de 150 espécies com distribuição pantropical (Grear 1970). Nas Américas, foram registradas 40 espécies (Lewis et al. 2005), das quais 35 ocorrem no Brasil (Fortuna-Perez et al. 2013, 2017, 2018; Cândido et al. 2014, 2016, 2018, 2019), habitando ambientes com padrões sazonais de inundação e fogo, bem como áreas perturbadas de solos pobres, entre áreas florestadas e campos abertos (Grear 1970). Esta espécie é comumente encontrada em áreas de cerrado, onde podem apresentar órgão subterrâneo espessado, uma adaptação xerofítica típica de ambientes savânicos.

Algumas espécies de *Eriosema* apresentam propriedades medicinais e são utilizadas na medicina popular para tratamento de disfunção erétil e/ou impotência sexual (Ojewole 2007), tosse (Kokwaro 2009), doenças de pele (Awouafack et al. 2013a), no combate à infecções microbianas (Awouafack et al. 2008, 2013b), infertilidade e doenças ginecológicas como menopausa (Burkill 1985), para diarreia, desintoxicação e hidrofobia (Ma et al. 1998), laxante e anti-inflamatório (Hirschmann & De Arias 1990; Rodrigues & Carvalho 2001; Santos et al. 2016).

Considerando a importância e ampla distribuição de *Eriosema*, avaliamos que este gênero de plantas representa um bom modelo para investigar em detalhe as fibras gelatinosas e verificar possíveis variações na estrutura da parede celular e na abundância

dessas células especializadas nos diferentes órgãos, da mesma espécie e entre espécies do gênero, contribuindo para o entendimento do papel adaptativo dessas células. Neste estudo investigamos comparativamente as fibras gelatinosas no eixo vegetativo aéreo e subterrâneo de táxons de *Eriosema* com o objetivo de i) acessar variações na distribuição e organização das fibras gelatinosas, na estrutura anatômica e na histoquímica da parede celular, dentro e entre táxons, buscando associar tais variações com possíveis funções; ii) estudar o desenvolvimento das fibras gelatinosas focando nos processos celulares de síntese e composição das camadas parietais visando a compreender as variações na arquitetura das paredes celulares.

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Primeiro capítulo

"Variations in the architecture and histochemistry of the gelatinous fibers across the vegetative axis of *Eriosema* (DC.) Desv. (Leguminosae) species from Brazilian Cerrado"

Este capítulo foi submetido para a revista *Flora* no dia 22 de dezembro de 2019 (comprovante em anexo)

PRIMEIRO CAPÍTULO

Variations in the architecture and histochemistry of the gelatinous of *Eriosema* (DC.) Desv. (Leguminosae) species from the Brazilian Cerrado

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Declarations of interest: none.

Abstract

G-fibers have been widely studied in tension wood, but few studies are available on shrub and sub-shrub plants, specially comparing aerial and underground organs of the same plant. The goal of this study is to investigate comparatively the general pattern of G-fibers distribution, and the structure and chemistry of G-fibers walls between aerial and underground organs in 19 *Eriosema* taxa occurring in savanna ecosystems, locally called cerrado, in order to provide evidence for possible functional diversity of G-fibers. The samples were processed according to standard techniques in plant anatomy and histochemistry. G-fibers occurred throughout the vegetative body and were exclusively extraxillary in organs with primary growth and extraxyllary and xyllary in organs with incipient secondary growth. G-fibers were more abundant and had a thicker G-layer in the underground organs. In all the organs analyzed, the G fibers were identified by the presence of a non-lignified internal translucent layer (G layer), with an irregular contour sometimes occupying the entire lumen of the fiber. The abundance and wide occurrence of G-fibers in the vegetative axis of *Eriosema* plants may represent an adaptive mechanism associated with water reserve and mechanical functions. We suggest that the conditions of the savanna environment can stimulate the differentiation of gelatinous fibers in these plants. In addition the ecophysiological significance of the G-fibers, we emphasize their taxonomic value as a unifying character of the *Eriosema* genus.

Keywords Cell wall architecture, Gelatinous fibers, Gelatinous layer, functional significance, vegetative axis and underground organs

Introduction

Gelatinous fibers (G-fibers) exhibit a highly modified internal layer of translucent appearance, rich in crystalline cellulose and usually devoid of lignin, called gelatinous layer (Dickison, 2000; Evert 2006; Clair et al., 2008; Ruelle, 2014) or tertiary cell wall (Gorshkova et al., 2018). The literature shows conflicting reports about the presence of lignin in the G-layer (Joseleau et al., 2004; Gierlinger and Schwanninger, 2006; Lehringer et al., 2008), as well as regarding the nature of this layer. For some authors, this layer is part of the secondary cell wall (Clair et al., 2018), while for others, because the G-layers are developed after the primary and secondary cell walls, have a distinct composition, architecture (Mellerowicz and Gorshkova, 2012) and physical properties is considered a tertiary cell wall (Gorshkova et al., 2018).

In general, mature G-fibers exhibit three distinct layers: the middle lamella and primary cell wall (P), secondary walls (S) with a variable number of lignified layers (S_1-S_3), and the inner, non-lignin or low lignin G-layer (G). Variations in the secondary layers have been reported, with three types usually described: $S_1 + S_2 + S_3 + G$; $S_1 + S_2 + G$; $S_1 + G$ (Singh and Donaldson, 1999; Donaldson and Turner, 2001). Interspecific variations in the number of the S-layers in G-fibers and even in the presence/absence of the G-layer in relation to plant ecophysiological conditions were reported (Rôças et al., 1997; Pramod et al., 2014; Piva et al., 2019).

G-fibers were initially examined in the tension wood of eudicotyledonous trees, receiving the name of "fibers of reaction", where they showed a stress response that allowed movement of mature organs and reinforced their structure and stability (Mellerowicz and Gorshkova, 2012). However, G-fibers have been found in several plant organs in primary or incipient secondary growth, occurring in both vascular and non-vascular tissues (Tomlinson, 2003; Gorshkova and Morvan, 2006; Toghraie et al., 2006;

Gorshkova et al., 2018). Therefore, diverse roles have been assigned to G-fibers in addition to generating tensional force under stress.

The wide occurrence of G-fibers in different groups of vascular plants indicates that their specialization arose at an early stage in the evolution of terrestrial plants (Mellerowicz and Gorshkova, 2012) and represents a strategy for survival in adverse environmental conditions (Böchek and Lyshede, 1972; Schreiber et al., 2010). For example, G-fibers may act as water storage sites, thus playing an important physiological role for plants subjected to water deficits and temperature extremes (Lyshede, 1978; Paviani, 1974, 1978; Marcati et al., 2001; Piva et al., 2019). Aluminum compartmentalization was detected in the G-layer in Melastomataceae plants living in soils rich in this element (Milanez et al., 2017). Additionally, the mechanical role of the G-fibers has been highlighted in ecosystems subjected to high irradiance (Sperry, 1982; Fisher and Stevenson, 1981) and in fire-prone environments, under the effect of desiccation (Fisher, 2008). However, there are very few studies available on the in the underground organs and its comparison to aerial organs. Thus, the supposed role of G-fibers in aerial and underground organs, whether similar or not, is an important issue that needs detailed investigation to unravel the functional diversity of G fibers in the entire plant.

Eriosema (DC.) Desv. is a pantropical genus of Leguminosae, inhabiting environments and regions that experience seasonal flooding and fire, as well as disturbed areas, mainly in open fields (Grear, 1970). In Brazil, there are approximately 35 species widely distributed in the Brazilian Cerrado (Fortuna-Perez et al., 2018; Cândido et al., 2019), a Neotropical savanna. The presence of thickened underground organs is one of the most important characteristics of *Eriosema* species, as this distinguishes this genus inside the subtribe Cajaninae in Brazil (personal observation Fortuna-Perez AP). Previous

studies have been reported the occurrence of G-fibers in leaves of the *Eriosema* species (Piva et al., 2019).

Here, we investigate comparatively the general pattern of G-fibers distribution, and the structure and chemistry of G-fibers walls between aerial and underground organs in different species of the *Eriosema* in order to provide evidence for possible functional diversity of G-fibers.

Materials and methods

Sample collection

This study was conducted on 19 taxa of *Eriosema*, six of which were obtained from national and foreign herbaria while the others were collected in the field (Table 1 – supplem.). All specimens were obtained from the Brazilian Cerrado areas. Vouchers were deposited in the "Irina Delanova de Gemtchujnicóv" Herbarium (BOTU).

The cerrado is a mosaic of vegetation consisting of the following physiognomies: "*campo limpo*" (open grassland), "*campo sujo*" (bushed grassland), "*campo cerrado*" (wooded grassland), *"campo rupestre"* (rocky fields), "*canga*" (ironstone), "*cerrado sensu stricto*" (woodland) and "*cerradão*" (a type of seasonal forest). Generally, this ecosystem is characterized by a seasonal climate, distinct rainy summers and dry winters, intense solar irradiation, and frequent fires. Soils are deep, well-drained, acidic, and have extremely low nutrient and high aluminum concentrations (Ratter et al., 1997; Oliveira and Marquis, 2002).

Sample processing and anatomical studies

The leaf (terminal leaflet), stem (primary and transition from primary to secondary growth) and underground organ of three individuals per species were examined (Figure 1). Leaves, which were selected from the third node, were analyzed at the petiole and midrib region. Stems were analyzed from the apical region and median region of internodes. Underground organ was analyzed the thickest region of the organ.

To examine the anatomy of terminal leaflets, herbalized samples were placed in deionized water on a hot plate (50°C) for 10 minutes, treated with 2% KOH solution for 2 h, washed several times with deionized water (Smith and Smith, 1942), and stored in 70% alcohol.

Fresh specimens were fixed in FAA50 (Johansen, 1940) and stored in 70% alcohol. A portion of the sample (fresh and herbalized) was sectioned using a Ranvier's microtome (about 10 μm thickness). Cross sections were clarified in 50% sodium hypochlorite, stained with 1% aqueous solution of Astra Blue and Safranin (Kraus and Arduin, 1997), and mounted on semipermanent slides in 50% glycerin. To detect lignin, cross sections of fresh samples were treated with acidified phloroglucine (Sass, 1951). Additional sections from each sample were dehydrated in an alcoholic series and infiltrated in historesin (Leica Historesin®). Cross sections (5 μm thickness) were obtained using a rotary microtome (RM2255 Leica) with a type C steel knife, stained with 0.05% Toluidine Blue and pH 4.7 (O'Brein et al., 1964 - modified), and permanently mounted on Entellan®.

Morphological classification of the underground systems followed Appezzato-da-Glória (2015). Samples were cross-sectioned on a sliding microtome (Reichert 335755), clarified in 50% sodium hypochlorite, stained with 1% aqueous solution of Astra blue

(Roeser, 1972) and Safranin (Kraus and Arduin, 1997), dehydrated in alcoholic series and butyl acetate, and permanently mounted on Entellan®.

Observation and photo documentation were performed using an optical microscope (Leica DMR) coupled to a Leica DFC 425 image capture system with projection of the corresponding micrometric scales.

Distribution and organization of G-fibers

Classification of the distribution and organization of G-fibers was based in the description to typical sclerenchyma fibers (Evert, 2013). Regarding the distribution, fibers were classified as: i) extraxylary fibers (when located outside the xylem) or ii) xylary fibers (when located in the xylem). For organization, fibers were classified as: i) segmented cylindrical strands in stem (Figure 2A), ii) complete cylindrical strands in stem (Figure 2B), iii) complete sheath around the vascular bundle in midrib (Figure 2C), iv) caps associated with vascular bundles in midrib (Figure 2D) and v) groups in the parenchyma in underground organ (Figure 2E).

Qualitative analysis

Measurements were performed on photomicrographs of the cross sections obtained from midrib, internode and subterranean organ (root or stem) using the ImageJ 1.43a version 64 program.

Values were expressed as the percentage of G-fibers per 1 mm^2 in the midrib (mean of three individuals for each species, totalizing 19 taxa, Table 1 - supplem.). Midribs were chosen due to a greater availability among individuals and collection sites.

The thickness (µm) of the walls (primary + secondary = P + S, and tertiary = G) was measured in three individuals of each taxon. For this analysis, were selected 10 taxa

that presented leaf, aerial stem and thickened underground organ. Values were expressed as average of the three measurements for each organ, in each taxon.

Results

In this study, we compared the general pattern of G-fibers distribution, and the structure and chemistry of G-fibers walls between aerial and thickened underground organs of 19 *Eriosema* taxa. We examined the chemistry, structure, and arrangement of cell walls, with emphasis on the G-layer.

Distribution and organization of G-fibers

In leaves, G-fibers were exclusively extraxylary (Figure 3A-B). In midribs, G-fibers were in caps associated with the phloem in the abaxial surface and occurred in small groups on the adaxial surface (Figure 3A). In the petiole of all taxa, G-fibers were arranged in caps on the periphery of the phloem (Figure 3B). In all taxa, G-fibers occurred adjacent to typical sclerenchyma fibers (Figure 3C).

The area occupied by G-fibers in the midrib varied between taxa, ranging from 0.50% to 9.59% per mm². The highest density of G-fibers occurred in *Eriosema glabrum*, *E. floribundum*, and *E. congestum*, while the lowest concentration was observed in *E. laxiflorum* (Figure 4).

In general, when comparing the thickness of the G-fiber walls between midrib, stem and underground organs, the median thickness of the P+S walls was similar between the organs (0,77µm; 0,89µm; 0,98µm, respectively), although thicker walls (P+S+G) were recorded in the midrib. A thicker G-layer (tertiary wall) was registered in the underground organs (Table 2).

G-fibers were extraxylary in the apical region of the stem and presented in complete cylindrical strands for all taxa analyzed (Figure 3D). In the internode region, G-fibers were extraxylary and xylary (Figure 3E) except in *E. defoliatum* samples presenting immature fibers with evident cellular content (Figure 3F). Extraxylary G-fibers were organized into complete cylindrical strands (Figure 3D), while xylary fibers occurred in groups separated by parenchyma rays (Figure 3G-H).

Underground organs of *Eriosema* varied from xylopodium (of unknown origin) bound with tuberous roots (*E. crinitum* var. *crinitum* and *E. glabrum*) (Figure 5A), roots with tuberous and non-tuberous portions (*E. congestum* and *E. macrostipulatum*) (Figure 5B-C), horizontal growth gemmiferous roots (*E. heterophyllum*) (Figure 5D), slightly thickened roots (*E. brevipes, E. floribundum, E. hatshbachii*, and *E. simplicifolium* var. *simplicifolium*) (Figure 5E), and tuberous roots (*E. campestre* var. *campestre, E. campestre* var. *macrophyllum*, and *E. irwinii*) (Figure 5F). In all taxa except *E. irwinii*, vascular parenchyma was filled with starch grains. In the thickened proximal region of the underground organs of all analyzed taxa, except *E. irwinii*, G-fibers were organized in tangential bands separated by parenchyma rays (Figure 3I), concentrated in small groups in the central region (Figure 3J), and usually filled with starch grains (Figure 3K).

Anatomy and chemistry of G-fibers

In all analyzed organs, G-fibers had an inner layer (G-layer) of irregular contour (Figure 6A - arrowhead) and were thicker than the other layers. The G-layer, which is naturally translucent, turned blue when stained with Astra Blue (Figure 6A) and purplish-blue when stained with Toluidine Blue (Figure 6B), indicating the presence of cellulose. The P-layer, which comprised the middle lamella and primary wall, was relatively thin (sometimes imperceptible), while the S-layer showed variable thickness. Both layers (P

+ S) turned red when stained with Safranin (Figure 6A) and bluish green when stained with Toluidine Blue (Figure 6B), indicating the presence of lignin. Thus, the lignin test was positive in the S-layers of G-fibers in all analyzed organs (Figure 6C-D) and lignification was not detected in the G-layer (Figure 6C) (Table 3).

Discussion

In this study, using standard methods in plant anatomy and histochemistry we studied the general pattern of G-fibers distribution, and the structure and chemistry of G-fibers walls between aerial and underground organs in 19 taxa of *Eriosema* in order to provide evidence for possible functional diversity of G-fibers.

All the studied taxa presented G-fibers in the aerial and underground organs, except *Eriosema defoliatum* that didn't have G-fibers in the stem. The absence of G-fibers may be associated with the growing conditions of this species, which is deciduous and at the time of its collection, it was in the regrowth phase, therefore with very young stems.

In general, G-fibers were more abundant and exhibited thicker G-layer in the underground organs (root and/or stem) in relation to the aerial organs. On the other hand, G-fibers with lignified thicker walls (P+S) were recorded in the midrib. These variations observed between aerial and underground organs are probably related to the impact of environmental factors, which is different on the root, stem and leaves. In this sense, wind and gravity are climatic factors that directly affect the development of supporting tissues in aerial organs (Esau, 1977; Evert, 2006). In fact, G-layer and collenchyma cell walls are similar in wall structure (thick nonlignified walls) and conditions of the protoplast, and both develops in organs or plant parts subjected to mechanical stresses, such as wind, attachment of weights to inclined shoots and spatial position of their axes in response to

environmental signals (Esau, 1977; Evert, 2006). In this study, differently of the expected, G- layer are thicker in the underground organs, which are, in some way, protected from climatic factors. Besides the environmental conditions, endogenous factors, such as levels of auxins and gibberellic acid have a significant effect on the differentiation of the cell walls of the supporting tissues (Lev-Yadun, 1997; Evert, 2006).

The location of G-fibers did not vary among *Eriosema* taxa or individuals of the same taxon. G-fibers were always associated with the vascular system, as they were extraxylary and/or xylary and occurred adjacent to typical sclerenchyma fibers. Similar G-fiber distributions have been described in organs of eudicots showing primary and secondary growth (Zimmermann et al., 1968; Jourez, 1997; Tomlinson, 2003; Gorshkova and Morvan, 2006; Toghraie et al., 2006; Fisher, 2008; Bowling and Vaughn, 2008; Carlquist, 2014).

The organization of G-fibers varies among organs, especially in roots (Patel, 1964). In the present study, G-fibers occurred predominantly in complete cylindrical strands, while others occurred in groups in organs with primary growth or in tangential bands separated by rays of parenchyma in organs with secondary growth. Anatomical studies have shown that the subterranean system of *Eriosema* plants is complex, consisting of roots (tuberoses and gemmiferous) or a combination of thick root and xylopodium (of unknown nature to date). Therefore, the ability of these plants to regrow is directly associated with the presence of bud-carrying underground organs (Rizzini, 1965), abundant starch grains (Appezzato-da-Glória, 2003), and G-fibers (Fisher, 2008). Thus, presence of G-fibers in these organs may contribute to the survival of these plants. In fact, all the studied species of *Eriosema* occur in savanna ecosystems characterized by high periods of water deficit, high luminous intensity, soils with low nutrient availability (with excess of aluminum in the savanna and iron in the iron stone) and subject to winds

and recurrent fires (Coutinho, 1978, 2002; Sarmiento et al., 1985). Therefore, the G-layer can act as a temporary water reservoir, as suggested for *Plathymenia reticulata* leaves, a legume from the Brazilian Cerrado (Paviani, 1974; Paviani and Ferreira, 1974; Paviani, 1978).

Since other polysaccharides occur in the G-layer, in addition to cellulose, which impart hygroscopic and contractile properties to this layer (Bowling and Vaughn, 2008), the G-layer has the capacity to absorb a large amount of water and become swollen, occupying the entire cell lumen (Evert, 2006). The effect of water on cellulose structure and its physical properties have been widely investigated, and results suggest that the change in the state of water in cellulose (during dehydration and rehydration) is important to its structural and mechanical properties (Fang and Catchmark, 2014). The hygroscopic swelling of the G-layer could lead to the contraction of the G-fiber in situations of dehydration (Fang and Catchmark, 2014). Based on statements, we suggest that G-fibers may play an important mechanical function in the plants studied, both in the aerial and underground axis. In this sense, G-fibers can contribute to maintaining leaf orientation in relation to the severity and position of the sun (Sperry, 1982) and in the upright position of developing stems (Fisher and Stevenson, 1981). In addition, G-fibers in environments prone to fire, as is the case of the cerrado, under the effect of desiccation, would allow the contraction and flexion of the hypocotyl and thickened underground organs, reducing damage to the buds (Fisher, 2008).

The G-layer, which was only been found in G-fibers, has been described in several species of Leguminosae (Gorshkova et al., 2010; Ghislain and Clair, 2017). Characteristics of the G-layer (higher thickness and non-lignification) were common in the different *Eriosema* taxa studied for all analyzed organs. Data from this study showed that lignin was always present in the S-layer in a similar pattern described in

sclerenchyma fibers of different species (Wahlgren, 1957; Wardrop, 1964; Saiki and Ono, 1971; Araki et al., 1982; Gierlinger and Schwanninger, 2006; Lehringer et al., 2008; Lehringer et al., 2009). The absence of lignification of the G-layer in all taxa studied is consistent with studies reporting zero or low lignin content in the G-layer in different species (Pilate et al., 2004; Meloche et al., 2007; Gorshkova et al., 2010; Schreiber et al., 2010; Mellerowicz and Gorshkova, 2012; Pramod et al., 2014). The very peculiar polysaccharide composition and the orientation of cellulose microfibrils in the G-layer may influence lignin deposition (Pilate et al., 2004).

In this study, G-fiber abundance varied considerably among *Eriosema* species and between organs of the same species. Variation in the abundance of G-fibers in aerial organs of related species was reported by Gorshkova and Mellerowicz (2012), and this variation may be associated with climatic and edaphic factors and mechanical stress to which the *Eriosema* plants are subjected. The effect (direct or indirect) of environmental factors, such as high temperatures, high solar radiation, frequent fires, and dystrophic soils on the ontogenesis and differentiation of plant tissues has been well documented (Guerra and Scremin-Dias, 2017). In fact, the association between high luminous intensity and presence of G-fibers with hygroscopic walls along the midrib was observed when comparing the leaf plasticity of *Alchornea triplinervia* under different light regimes and moisture gradients in the Atlantic Forest (Rôças et al., 1997). These studies reinforce the hypothesis on the adaptive role of the G-fibers and of the influence of the environmental factors on their differentiation (Böchek and Lyshede, 1972; Fisher, 2008; Schreiber et al., 2010). Thus, the environmental conditions of the cerrado may have improved the differentiation of G-fibers in the vegetative axis of *Eriosema*. The presence of G-fibers along the vegetative axis of the *Eriosema* plants, in addition the thickened underground organs, may explain why plants in this genus are able to inhabit

environments characterized by high luminosity, low relative humidity, winds and fires, as is typical of the savanna ecosystems. However, it is necessary to examine the same species cultivated under different soil and climate conditions to better understand this relationship.

Conflicts of interest

The authors declare no conflict of interest in relation to this publication.

Acknowledgments

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001 (Process 1585537) (scholarship to the first author), 'Conselho Nacional de Desenvolvimento Científico e Tecnológico'- CNPq for grant to Dr. Silvia Rodrigues Machado (Process 02657/2011-8, Edital MCT/CNPq Process 470649/2008-9 and 400567/2016-4), CNPq for doctoral scholarships to Wanderleia de Vargas (process 140598/2015-4), FAPESP for grants to Dr. Ana Paula Fortuna Perez (Process 2015/13386-0), Professor Dr. Beatriz Appezzato da Glória for help with the underground organs determination, and to the staff of the Laboratório de Anatomia da Madeira (FCA/UNESP).

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Appendices

Tables

Table 1 - Taxes studied, voucher specimen(s) and provenance of collected material

Taxa	**Voucher specimen(s)**	**Origin**	**Environment**
***Eriosema brevipes* Grear**	AP Fortuna-Perez et al. 2101 (BOTU)	Fresh	"rocky fields"
	D Villarroel et al. 2891 (UB)	Herbalized	"grassland"
	RS Rodrigues et al. 1434 (UEC)	Herbalized	"rocky fields"
Eriosema campestre* Benth. var. *campestre	AP Fortuna-Perez et al. 3334 (BOTU)	Fresh	"seasonal forest"
	W Vargas 70 (BOTU)	Herbalized	"grassland"
	A Pott and V Pott 11959 (CGMS)	Herbalized	"grassland"
***Eriosema campestre* var. *macrophyllum* (Grear) Fortunato**	TC Piva 78 (BOTU)	Fresh	"woodland bushed"
	AP Fortuna-Perez et al. 3336 (BOTU)	Fresh	"woodland bushed"
	AP Fortuna-Perez et al. 5006 (BOTU)	Fresh	"ironstone"
***Eriosema congestum* Benth.**	AP Fortuna-Perez et al. 3335 (BOTU)	Fresh	"woodland bushed"
	MJ Silva 7396 (UFG)	Herbalized	"rocky fields"

	L Coradin 4258 (UEC)	Herbalized	“woodland”
Eriosema crinitum* (Kunth) G.Don var. *crinitum	W Vargas et al. 100 (BOTU)	Fresh	“woodland bushed”
	AP Fortuna-Perez et al. 5005 (BOTU)	Fresh	“ironstone”
	ES Cândido et al. 1075 (OUPR)	Herbalized	“rocky fields”
***Eriosema crinitum* var. *pulchellum* Benth.**	A Pott and V Pott 13974 (CGMS)	Herbalized	“wooded grassland”
	W Mantovani 1177 (SP)	Herbalized	“rocky fields”
	HS Irwin et al. 21971 (SP)	Herbalized	“grassland”
***Eriosema defoliatum* Benth.**	AP Fortuna-Perez et al. 3333 (BOTU)	Fresh	“wooded grassland”
	ES Cândido 1500 (BOTU)	Herbalized	“rocky fields”
	MV Martins et al. 85 (CEN)	Herbalized	“woodland”
***Eriosema elegans* Fort.-Perez & M.J. Silva**	AP Fortuna-Perez et al. 2655 (BOTU)	Fresh	“wooded grassland”
	MJ Silva 7228 (UFG)	Herbalized	“rocky fields”
	AP Fortuna-Perez et al. 2000 (BOTU)	Herbalized	“rocky fields”
***Eriosema floribundum* Benth.**	AP Fortuna-Perez et al. 5000 (BOTU)	Fresh	“rocky fields”
	ES Cândido et al. 1000 (OUPR)	Herbalized	“woodland”
	ES Cândido et al. 1090 (OUPR)	Herbalized	“rocky fields”
***Eriosema glabrum* Mart. ex Benth.**	TC Monteiro 67 (BOTU)	Fresh	“ironstone”

	ES Cândido et al. 1067 (OUPR)	Herbalized	“grassland”
	RF Viera 1646 (CEN)	Herbalized	“rocky fields”
***Eriosema hatschbachii* Fort.-Perez & G. P. Lewis**	AP Fortuna-Perez et al. 5004 (BOTU)	Fresh	“woodland bushed”
	G Hatschbach and R Kummrow 49596 (US)	Herbalized	“rocky fields”
	AP Fortuna-Perez et al. 1401 (OUPR)	Herbalized	“woodland bushed”
***Eriosema heterophyllum* Benth.**	AP Fortuna-Perez et al. 5003 (BOTU)	Fresh	“rocky fields”
	L Coradin 7370 (CEN)	Herbalized	“woodland”
	ES Cândido et al. 1068 (OUPR)	Herbalized	“grassland”
***Eriosema irwinii* Grear**	ES Cândido et al. 1106 (BOTU)	Fresh	“woodland bushed”
	TSM Grandi et al. 1173 (BHCB)	Herbalized	“woodland”
	AP Fortuna-Perez et al. 1467 (OUPR)	Herbalized	“woodland”
***Eriosema laxiflorum* Harms**	MJ Silva 4407 (UFG)	Herbalized	“rocky fields”
	APM Lira-Gouvêa and EF Alteff 293 (UB)	Herbalized	“grassland”
	APM Lira-Gouvêa and EF Alteff 294 (UB)	Herbalized	“grassland”
***Eriosema longifolium* Benth.**	AP Fortuna-Perez et al. 1437 (OUPR)	Herbalized	“woodland”

	AP Fortuna-Perez et al. 1442 (OUPR)	Herbalized	“grassland”
	MJ Silva 4025 (UFG)	Herbalized	“rocky fields”
Eriosema macrostipulatum **Fort.-Perez, Cândido & M.J. Silva**	AP Fortuna-Perez et al. 3448 (BOTU)	Fresh	“woodland”
	AP Fortuna-Perez et al. 5001 (BOTU)	Fresh	“wooded grassland”
	AP Fortuna-Perez 1447 (OUPR)	Herbalized	“woodland”
Eriosema pycnanthum var. pycnanthum **Benth.**	SG Rezende and MS Mendes 2088 (BHCB)	Herbalized	“woodland bushed”
	FF Carmo et al. 1957 (BHCB)	Herbalized	“ironstone”
	T Mansur et al. 198 (BHCB)	Herbalized	“rocky fields”
Eriosema simplicifolium **var. *micranthum* Grear**	RS Rodrigues et al. 1386 (UEC)	Herbalized	“woodland bushed”
	HS Irwin et al. 11428 (NY)	Herbalized	“grassland”
	W Vargas 76 (BOTU)	Herbalized	“woodland bushed”
Eriosema simplicifolium **(Kunth) G.Don var. *simplicifolium***	AP Fortuna-Perez et al. 2102 (BOTU)	Fresh	“seasonal forest”
	IS Miranda 757 (UEC)	Herbalized	“grassland”
	TC Piva 79 (BOTU)	Fresh	“woodland bushed”

Table 2 - Mean cell wall primary and secondary (P+S), and tertiary (G-layer) of gelatinous fibers in ten taxa of *Eriosema*.

Taxa	MIDRIB		STEM		UNDERGROUND ORGAN	
	P+S	**G**	**P+S**	**G**	**P+S**	**G**
E. brevipes	1,20	2,54	1,00	3,50	2,30	8,23
E. campestre var. campestre	0,86	1,67	0,97	2,20	0,91	3,86
E. campestre var. macrophyllum	0,90	5,66	0,73	3,52	0,89	5,53
E. congestum	0,82	5,44	0,59	4,62	0,65	6,17
E. crinitum var. crinitum	0,88	5,17	0,62	3,66	0,81	4,11
E. glabrum	0,96	3,94	0,65	5,32	0,88	4,75
E. hatshbachii	0,81	6,83	0,73	7,02	0,65	4,86
E. heterophyllum	1,66	2,88	1,01	3,90	0,46	4,50
E. macrostipulatum	0,75	3,96	0,51	3,18	0,67	5,77
E. simplicifolium var. simplicifolium	0,97	5,46	0,94	4,91	0,65	4,84

Table 3 – Histochemical test in fresh material for the detection of lignin in the G-layer (phloroglucine acidic). (Legends: P – petiole, MR – midrib, SA – stem apical region, SI – stem region of internodes, U – underground organ, + positive reaction, - negative reaction, no: not observed, 0: absent gelatinous fibers.

Taxa	**P**	**MR**	**SA**	**SI**	**U**
E. brevipes	-	-	-	-	-
E. campestre var. *campestre*	-	-	-	-	-
E. campestre var. *macrophyllum*	-	-	-	-	-
E. congestum	-	-	-	-	-
E. crinitum var. *crinitum*	-	-	-	-	-
E. defoliatum	-	-	0	0	no
E. elegans	-	-	-	-	no
E. floribundum	-	-	no	no	-
E. glabrum	-	-	-	-	-
E. hatschbachii	-	-	-	-	-
E. heterophyllum	no	-	-	-	-
E. irwinii	-	-	-	-	0
E. macrostipulatum	-	-	-	-	-
E. simplicifolium var. *simplicifolium*	-	-	-	-	-

Figures

Figure

Figure 1 - Sample collection scheme used in the study for each analyzed organ.

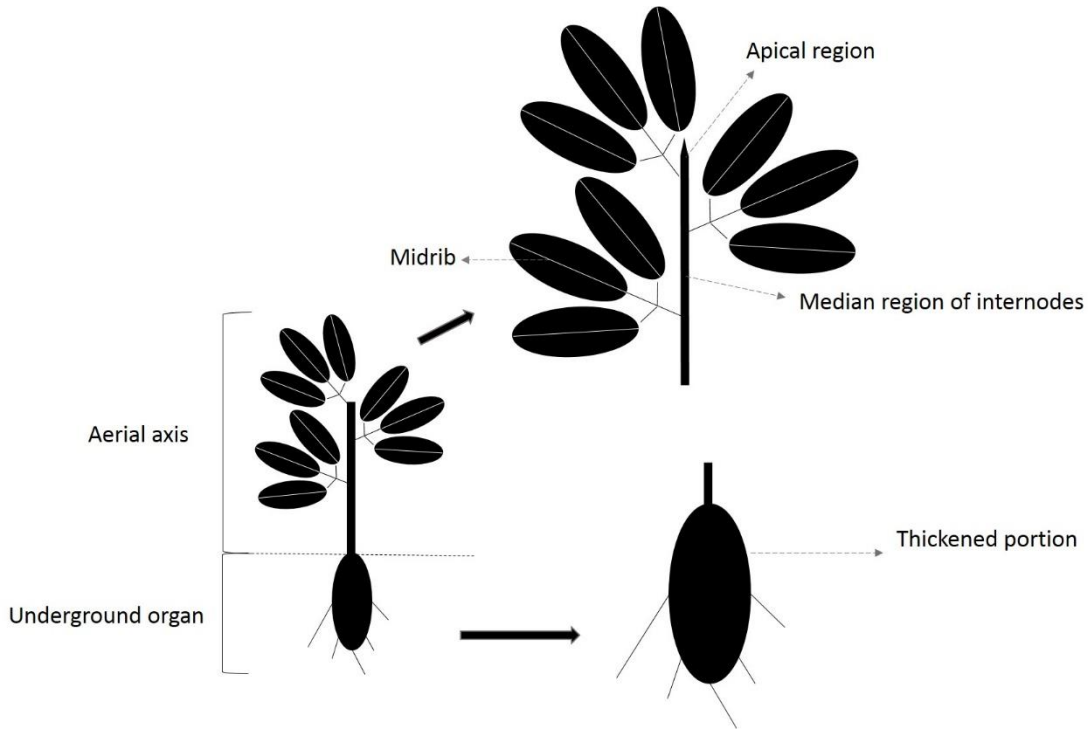


Figure 2 - Schemes to exemplify the organization of the gelatinous fibers (black trace), phloem (medium gray) and xylem (light gray) based on the description of Evert (2013). **A-B:** Stem, **C-D:** Midrib and **E:** Underground organ. **A –** segmented cylindrical strands, **B –** complete cylindrical strands, **C –** complete sheath around the vascular bundle, **D –** caps associated with vascular bundles and **E –** groups separated by parenchyma rays and xylem.

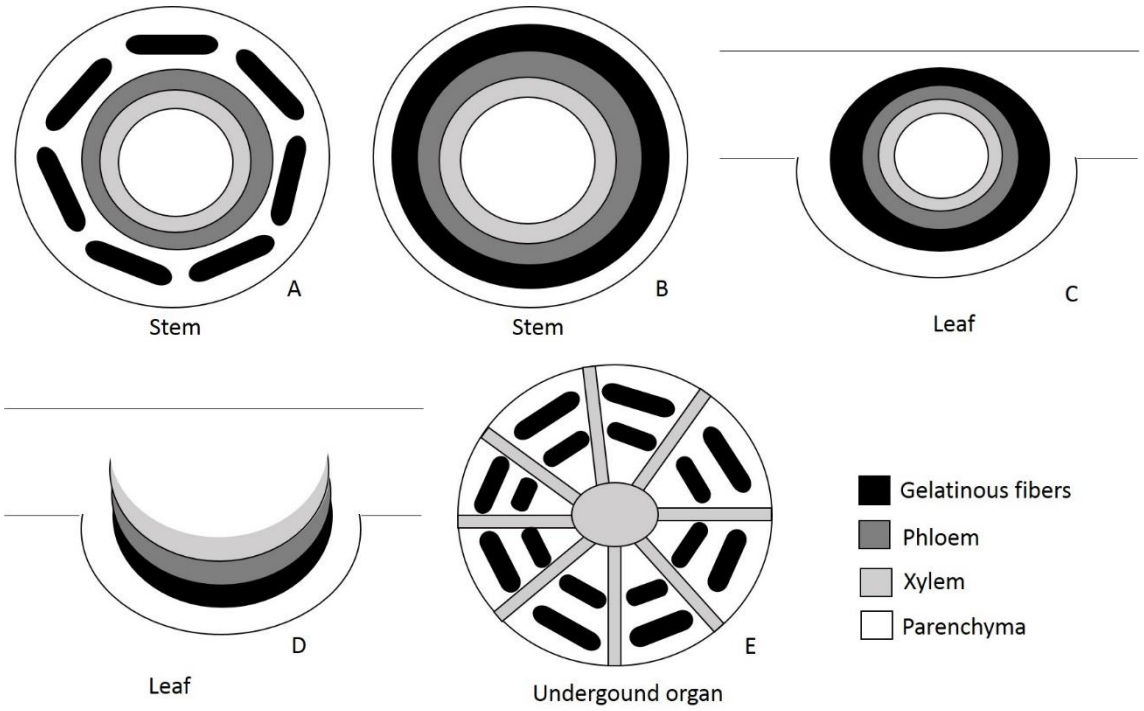
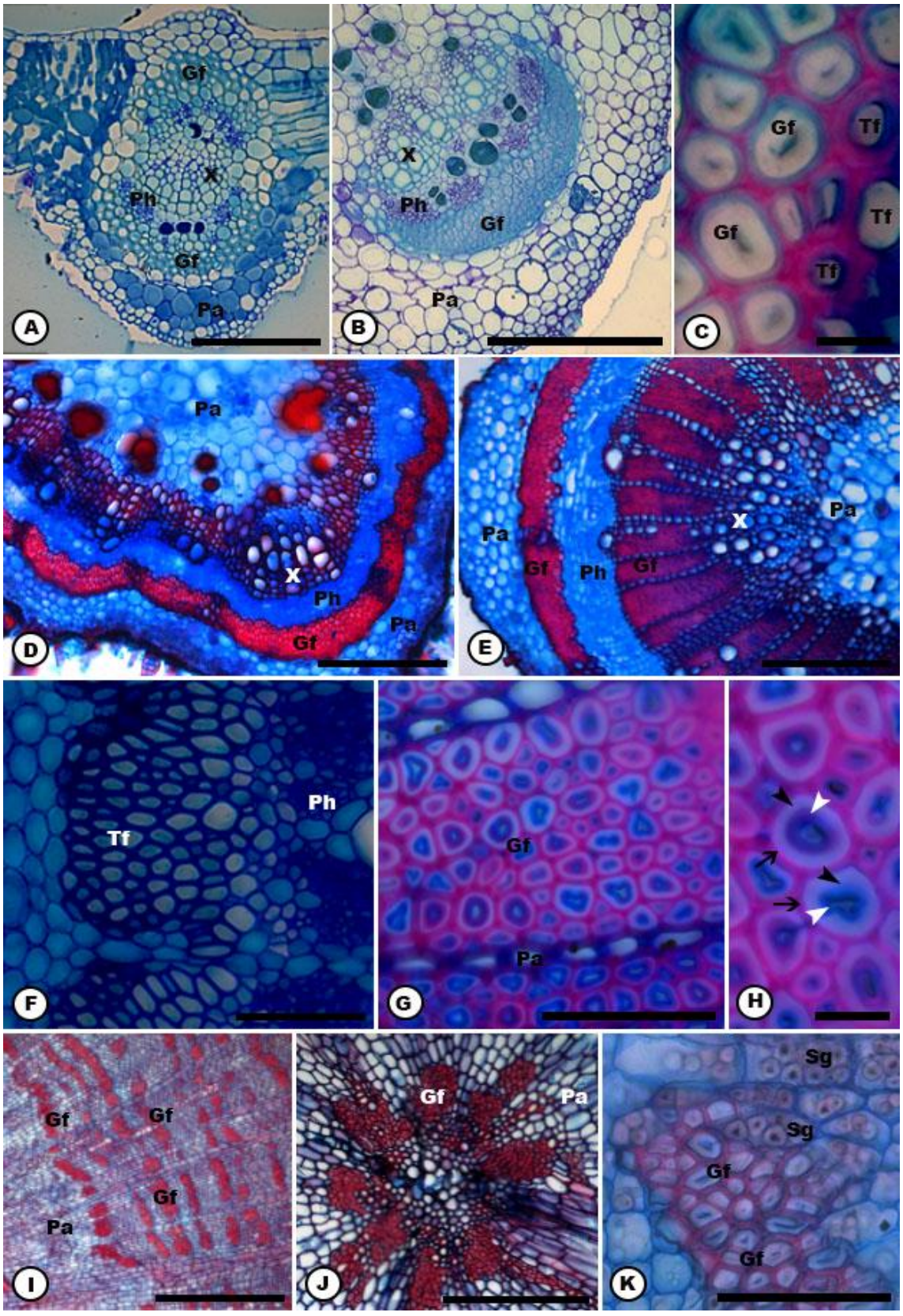


Figure 3 - Distribution and organization of G-fibers in taxa of the genus *Eriosema* in cross section. **A-B**: using a toluidine blue stain and **C-K:** using a safranin-blue strain. **A –** Overview of the midrib in *E. glabrum*. **B –** General view of the petiole *in E. campestre* var. *campestre.* **C –** Presence of G-fibers together with typical sclerenchyma fibers in the midrib of *E. crinitum* var. *crinitum*. **D –** General view of the extraxylary location of G-fibers organized into a complete cylindrical strand in the stem in the apical region of the *E. campestre* var. *campestre.* **E –** General view of the extraxylary and xylary location of G-fibers organized in complete cylindrical strands and in a group in the stem in the internodes region of *E. campestre* var. *campestre.* **F –** Stem in the internodes region of *E. defoliatum* where fibers are not gelatinous (no G-layer present). **G –** Xylary G-fibers in groups separated by rays of the parenchyma in the stem in the internodes region of *E. brevipes*. **H –** Highlight for the G-fibers, allowing the visualization of sublayers of the G-layer on the stem in the internodes region of *E. brevipes*. **I –** Details of the G-fibers occurring in bands in the underground organ of *E. simplicifolium* var. *simplicifolium*. **J –** Central region of the underground organ with the presence of G-fibers close to the protoxylem in *E. crinitum* var. *crinitum.* **K** – G-fibers in groups surrounded by parenchyma with starch grains in the underground organ of *E. campestre* var. *campestre*. **Captions:** Black arrowhead: G-layer, Black arrow: S-layer, Gf: G-fiber, Pa: Parenchyma, Ph: Phloem, Sg: Starch grains, Tf: Typical fibers, White arrowhead: inner layer the G-layer and X: Xylem. **Scale bars:** J = 500 μm; A, D, E, F and I = 200 μm; B = 100 μm; G and K = 50 μm; C = 20 μm and H = 10 μm.


Gf
X
Ph
Gf
Pa
A
X
Ph
Gf
Pa
B
Gf
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Gf
Pa
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Figure 4 - Graphic representation as the percentage of G-fibers per 1 mm² in the midrib (mean of three individuals for each species) of the *Eriosema* taxa.

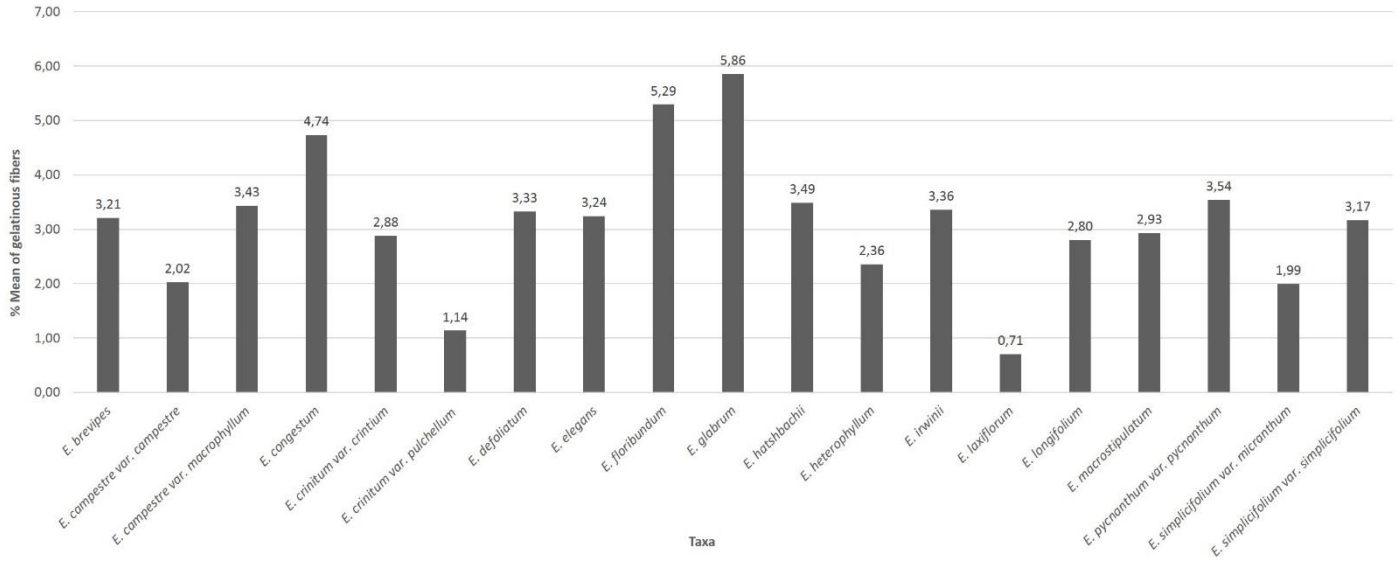


Figure 5 – Morphology of the underground organ of taxa of the genus *Eriosema* highlighting the region in which the analysed sample was taken. **A –** *Eriosema crinitum* var. *crinitum*. **B –** *E. congestum*. **C –** *E. macrostipulatum*. **D –** *E. heterophyllum*. **E –** *E. brevipes*. **F –** *E. irwinii*. **Scale bar:** 1cm

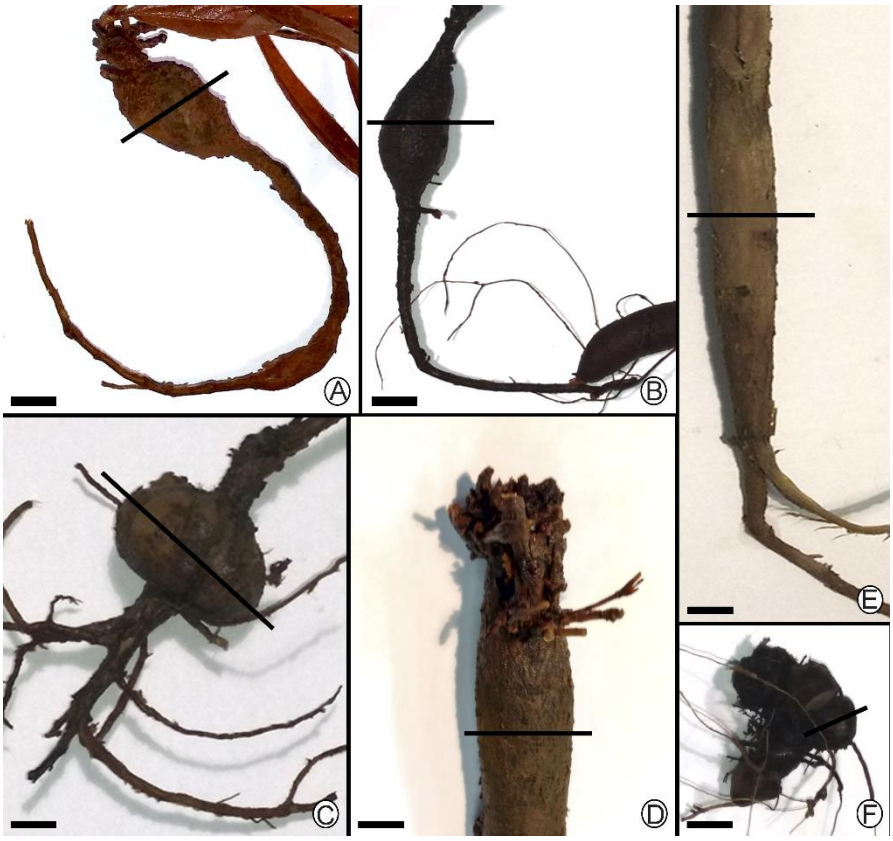
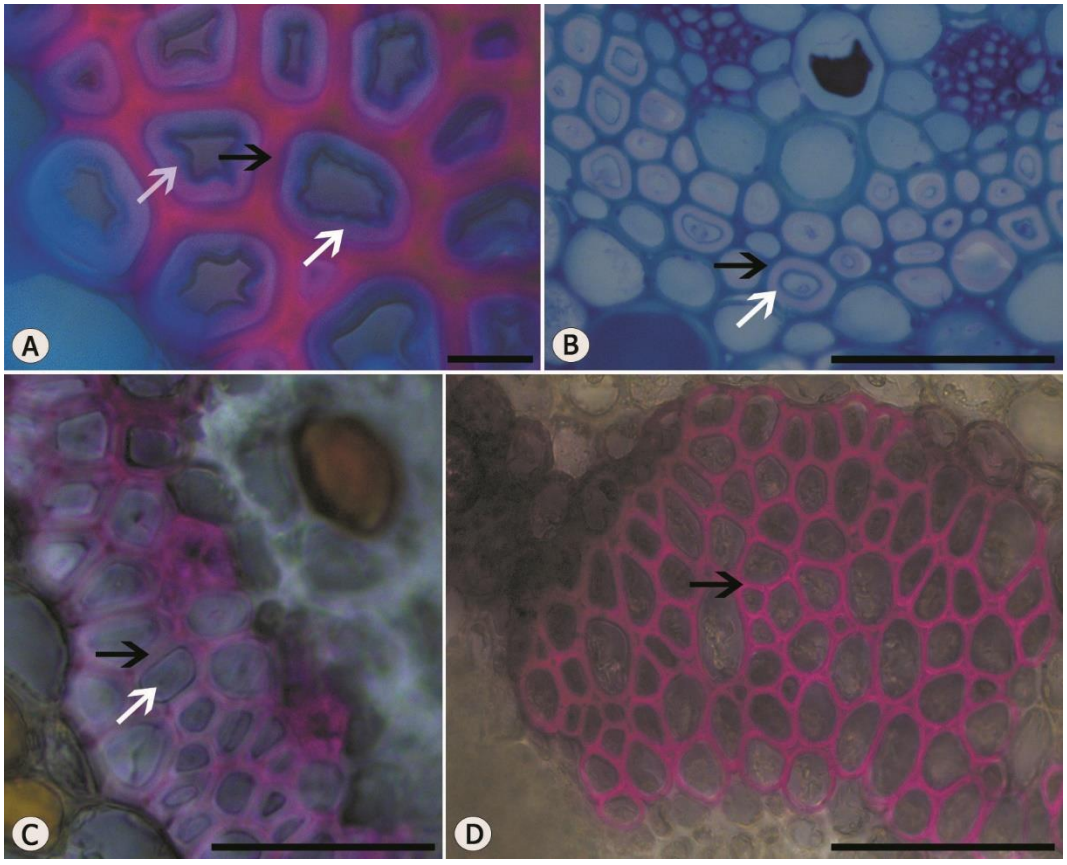


Figure 6 – Anatomy using safranin-blue and toluidine blue (A-B) and histochemistry using acidified phloroglucine (C-D) of fibers in the genus *Eriosema* in cross section. **A –** General characterization of G-fibers stained with Astra Blue and Safranin**. B –** General characterization of G-fibers stained with Toluidine blue. **C –** Histochemical test for lignin with a positive reaction at the P-layer and S-layer, but negative reaction of the G-layer in the stem of the *E. crinitum* var. *crinitum*. **D** – Histochemical test for lignin with a positive reaction at the P-layer and S-layer, and absence of G-layer in the stem of the *E. defoliatum*. **Captions:** Black arrow: S-layer and White arrow: G-layer. **Scale bars:** B-D = 50µm; A = 10µm.



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"Detection *in situ* of lignin, galactans and xylan study *in Eriosema simplicifolium* (Kunth) G.Don. (Leguminosae): A suitable approach for improve our understanding on gelatinous fibers constitution across the vegetative body"

Este capítulo foi escrito de acordo com as normas da revista *Planta* onde será submetido

SEGUNDO CAPÍTULO

Detection *in situ* of lignin and immunostaining study in *Eriosema simplicifolium* (Kunth) G.Don. (Leguminosae): An suitable approach for improve our understanding on gelatinous fibers constitution across the vegetative body

Abstract

Main conclusion **The organization and distribution of G-fibers and G-layer composition vary between organs of the same individual. The presence of G-layer lignification, characterizing the occurrence of composed G-layer, enables new interpretations for the physiological role of G-fibers.**

Gelatinous fibers (G-fibers) are a type of sclerenchyma cell and are thus named by the presence of an innermost layer on the secondary gelatinous appearance cell wall called the "gelatinous layer" (G-layer). The G-layer has been considered a tertiary cell wall because the composition and time of formation is different from the primary and secondary cell walls. Gelatinous fibers with composed G-layer the lamellar aspect featuring non-lignified layers and lignified layers (L-layer) interspersed were described in the tension wood and secondary phloem of some conifer and angiosperm species. However, variations in the architeture and chemical composition of the G-layer between species or diferent organs within the same species remain little known. The objective of this study was to inv*estigate the organization and composition of G-fibers in the vegetative axis of *Eriosema simplicifolium*, focusing on the G-layer. This study was conducted on leaves, stems (primary and secondary growth) and roots of this species using standard techniques of plant anatomy and histochemistry and immunolabeling methods for wall polysaccharides. G-fibers occurred throughout the vegetative axis of the plant, being extraxylary in the primary body and xylary in the stem and roots in secondary growth. The presence of composite G-layer was observed in the midrib, stem and roots in secondary growth. The occurrence of lignin and xylans in the secondary walls and L-layer of G-fibers was evidenced with the use of phloroglucine acidic and antibody LM10, respectively. Antibody LM5 marked the presence of galactans in the middle lamella, primary cell wall, single G-layer and non-lignified lamella of the composed G-layer. The presence of a lignified lamella in the G-layer would allow the G-fibers to act in the mechanical support of the aerial and/or underground organs, conferring resistance of these plants in adverse situations, such as drought, the passage of fire and herbivores, among other agents. On the other hand, G-fibers with G-layer non-lignified, therefore hydrophilic, may act in the temporary storage of water and other elements.

Keywords: Cerrado, G-fibers, G-layer, galactans, xylans

Introduction

Gelatinous fibers (G-fibers) is a type of sclerenchyma cell and is named by the presence of an innermost layer on the secondary cell wall, translucent and gelatinous in appearance, which is called a "gelatinous layer" (G-layer) (Evert 2013; Ruelle 2014). Due to G-layer was formed after the complete deposition of the secondary parietal layers (S-layer); some authors consider it a tertiary cell wall (Gorshkova et al. 2018a).

When compared to secondary cell walls, the G-layer exhibits specific characteristics such as the presence of high cellulosic content (80-90%), absence of xylan and lignin (Gorshkova et al. 2012; Mellerowicz and Gorshkova 2012) and high water content (Schreiber et al. 2010). Although the absence or low content of lignin in the G-layer is a reported feature in different species (Love et al. 1994; Gorshkova et al. 2000; Plomion et al. 2001; Pilate et al. 2004; Meloche et al. 2007; Bond et al. 2008; Kaku et al. 2009; Schreiber et al. 2010; Gorshkova et al. 2010; Mellerowicz and Gorshkova 2012; Pramod et al. 2014), lignin has been detected in the G-layer of G- fibers in tension wood *Populus deltoides* (Joseleau et al. 2004), *Acer*, *Fagus* e *Quercus* (Lehringer et al. 2008). Therefore, the occurrence of lignin in G-layer remains an arguable question.

Composed G-layer formed by a thick, non-lignified G-layer (G) interspersed with thin, lignified layer (L) forming concentric arrangements in cross sections, were described in xylem of the tension wood (Clair et al. 2006; Ruelle et al. 2007) and secondary phloem of conifers and eudicotyledons (Nanko 1979; Nakagawa et al. 2012, 2014). The number of repeats (*n*) of this arrangement [S1+S2+ *n* (G+L)] within the phloem fiber groups is variable (Nanko 1979; Nakagawa et al. 2012) and seems to be related to the developmental stage of the individual fibers (Nakagawa et al. 2014).

Immunohistochemical methods have enabled a better understanding of G-layer properties, specifically the polysaccharides components (Jones et al. 1997; Awano et al. 1998; Meloche et al. 2007; Bowling and Vaughn 2008; Ralet et al. 2010; Kim et al. 2010; Gorshkova et al. 2015; Chernova et al. 2018) as well as have shown promise for understanding cell wall dynamics during the development of G-fibers. In fact, immunolabeling techniques with specific antibodies have revealed other G-layer polysaccharide components such as galactose, rhamnogalacturonan I, arabinogalactan and proteins (Bowling and Vaughn 2008; Mikshina et al. 2013).

During a preliminary study of G-fibers in representatives of *Eriosema*, a pantropical genus of Fabaceae, we repeatedly found indicative occurrence of the lignin in G-layer of the *E. simplicifolium* (Kunth) G.Don. (Piva et al. unpublished), one specie widespread in the Brazilian *Cerrado* (a tropical savanna) in regions that experience seasonal fire.

The objective of this study was to investigate the organization and composition of G-fibers in the vegetative axis of *E. simplicifolium*, focusing on the G-layer. We considered that a combination of anatomical, histochemical and immunohistochemical analyses might provide support in understanding the constitution and functions of G-fibers in this species.

Materials and methods

Plant materials

Samples of the shoot apex, internodes, thickened primary root and fully expanded leaflets, were collected from three individuals of *Eriosema simplicifolium* (Kunth) G.Don growing in remnant of Cerrado *sensu stricto* (a Neotropical savanna) in the city of

Botucatu, district of Rubião Jr (22°53'35.9"S 48°29'23.6"W) state of São Paulo, Brazil. Vouchers were deposited in the Herbarium BOTU (Piva T.C. 79 - BOTU 33117)

Sample preparation

The samples were fixed for 48h in a FAA50 solution (formalin, acetic acid and 50% ethanol; 1:1:18 by volume) (Johansen, 1940) at room temperature and stored in 70% ethanol; the samples were dehydrated in an ethanol series and embedded in methacrylate resin (Historesin, Leica Microsystems, Nussloch, Heidelberg, Germany). Cross sections (5μm thick) were obtained using a semi-automated Leica RM2255 rotary microtome, stained with toluidine blue 0.05% in 0.1 mol L-1 phosphate buffer (Feder and O'Brien 1968), and sealed with Entellan (Merck KGaA, Darmstadt, Germany). Sections of the samples stocked in 70% alcohol were used to detect lignin with phloroglucine acidic (Sass 1951). Photographs were taken with a light microscope (Leica DMR) coupled to a digital camera (Leica DFC 425).

Immunolabeling

Samples (1mm) of leaves, stems and underground organ were fixed for 24h in 1% [v/v] glutaraldehyde and 4% [w/v] commercial formaldehyde in 0.1M phosphate buffer (pH 7.2) (McDowel and Trump 1976). Subsequently, the samples were dehydrated in an alcohol series, embedded in LR White resin (Hard Grade - London Resin Company) and polymerized in gelatin capsules at 50°C for 24h. Semi-thin (1μm) sections of samples were made with glass knives in a ultramicrotome (LKB Bromma, Ultrotome® III 8800). For immunolocalization, sections were (1) blocked with 0.1 m phosphate-buffered saline (PBS, pH 7.4) containing 3% (w/v) bovine serum albumin (BSA) for 1 h at room temperature; (2) incubated with one of the primary monoclonal antibodies LM5 and LM10 diluted 1 : 10 in PBS with 0.06% (w/v) BSA for 1 h at room temperature; and (3)

incubated with secondary anti-rat IgG linked to fluorescein isothiocyanate (FITC, Sigma-Aldrich) diluted 1 : 100 in PBS with 0.06% (w/v) BSA for 1 h at room temperature in the dark. The primary antibody treatment was omitted for the negative controls. At the end, the samples were washed three times in PBS for five minutes and mounted in CFM-1 solution (Electron Microscopic Sciences, Hatfield, PA, USA). Samples were viewed under a confocal laser scanning microscope (model LSM-510, Zeiss, Oberkochen, Germany) using an excitation wavelength of 488 nm and detection wavelengths between 505 and 530 nm.

Antibodies

LM5 and LM10 antibodies were select due to specific labeling for 1,4-beta-D-galactans and xylans, respectively. The LM5 antibody recognizes galactan epitopes (Jones et al. 1997), already described as generally absent from secondary cell walls (Andème-Onzighi et al. 2000) and present in the G-layer of fibers (Meloche et al. 2007). The LM10 antibody recognizes linear and low substitution xylans (McCartney et al. 2005), and has been reported that the presence of xylan in the cell wall is located in the layers where there is the presence of lignin, being a structure that facilitates lignification (Reis and Vian 2004; Nakagawa et al. 2014).

Results

The G-fibers occurred in all vegetative axis of *Eriosema simplicifolium*, being extraxylary in the primary body and xylary in the stem and root in secondary structure. In the leaves, the G-fibers formed caps on the dorsal surface of the midrib and small groups on the ventral surface (Figure 1A). In the primary structure stem, the extraxylary G-fibers were organized in a complete cylindrical strands around the phloem (Figure 1B), while in the secondary growth region the xylem fibers occurred in groups separated by

parenchymal rays (Figure 1C). In the thickened region of the primary root, with secondary growth, the G-fibers occurred in small groups distributed in tangential bands separated by parenchyma rays (Figure 1D).

Differentiated G-fibers exhibited three distinct walls: the middle lamella and the pectic-cellulosic primary cell wall (P), lignified secondary cell wall (S) with variation in the number of strata, and more internally, the G-layer (G). This layer, considered here as a tertiary cell wall, non-lignified (single G-layer) or partially lignified (composite G-layer) (Figure 1E). In the leaf (midrib region) (Figure 1F), stem (Figure 1G) and root (Figure 1H) regions in secondary growth, the composed G-layer presented a lignified internal lamella (L) interspersed with non-lignified lamella (G), (Figure 1E-H).

The presence of lignin in the secondary walls and in the L-layer of the composite G-fibers was evidenced with the use of phloroglucine acidic (Figure 2A-C). The LM10 antibody recognized xylans on the secondary cell wall and lignified lamella (L) of the composite G-layer (Figure 2D-F). Antibody LM5 marked the presence of galactans in the middle lamella, primary cell wall, single non-lignified G-layer and non-lignified lamella of the composite G-layer (Figure G-I). The results of immunolabelling analyzes are summarized in Table 1.

Discussion

Gelatinous fibers in *Eriosema simplicifolium* were associated with primary phloem and secondary xylem, as described in several taxonomic groups (Zimmermann et al. 1968; Jourez 1997; Tomlinson 2003; Gorshkova and Morvan 2006; Toghraie et al. 2006; Fisher 2008; Bowling and Vaughn 2009; Gorshkova et al. 2018a; Piva et al. 2019). In this species, the G-fibers were observed throughout the vegetative axis, which differs from preliminary studies on the distribution of these fibers. Many authors have described the presence of G-fibers only on the stems of different species (Liese 1926; Jagels 1963;

Patel 1964; Höster e Liese 1966; Fayle 1968; Zimmerman et al. 1968; Bhat and Karkkaihen 1981; Fisher 1982), while others reported gelatinous fibers only on the roots of Fabaceae species (Ranjani and Krishnamurthy 1987).

The arrangement of the G-fibers in the aerial vegetative axis of *E. simplicifolium* in bands and strands, corroborates what has already been described in the literature by representatives of Fabales, Sapindales, Myrtales and Urticales (Carlquist 2014). We suggest that the arrangement of the G-fibers in the secondary growth stem of *E. simplicifolium*, where they are located close to the parenchyma rays, would enable the direct involvement of the G-fibers with the substances carried by the radial parenchyma (Hosokawa et al. 2001; Barceló 2005).

The presence of lignin in the secondary cell walls of G-fibers, as observed in the present work, is a common feature of different plant species (Love et al. 1994; Gorshkova et al. 2000; Plomion et al. 2001; Pilate et al. 2004; Gorshkova et al. 2018b). Regarding the G-layer, in the present study we observed non-lignified G-layer in the G-fibers and composed G-layer in the G-fibers, that is, with lignified lamella interspersed with non-lignified lamella. The composite G-layer was restricted to the extraxylary in G-fibers of the midrib and xylary fibers of the secondary growing stem and root.

The use of immunolabeling with the LM10 antibody evidenced the presence of xylan in the lignified walls, both in the S and L layers; showed non-lignified in G-layer and P-layer (primary cell wall and middle lamella) staining in *E. simplicifolium*. Similar observation in G-fibers has already been described for Euphorbiaceae species. (Nakagawa et al. 2014). The presence of xylan in lignified cell walls (S-layer and L-layer) occurs due to the xylan host chemical structure that allows lignification (Reis and Vian 2004). The presence of galactans in the conventional G-layer evidenced with the LM5

antibody was also observed in *Liquidambar styraciflua*, *Celtis occidentalis* (Bowling and Vaughn 2008) and *Cannabis sativa* (Crônier et al. 2005), however, the composition of the G-layer may vary at inter and intraspecific level (Pramod et al. 2014) and present other components besides galactans, such as high cellulosic and proteins content (Lafarguette et al. 2004; Andersson-Gunnerås et al. 2006; Nishikubo et al. 2007; Bowling and Vaughn 2008; Kim and Daniel 2012; Mikshina et al. 2013).

The presence of xylan and lignin is related to the rigidity and resistance of the cell wall (Raes et al. 2003), being lignin the last component incorporated in the cell wall, thus allowing its strengthening (Fengel and Wegener 1984). Lignin deposition on cell walls results in decreased water content due to the filling of available spaces between polysaccharides (cellulose microfibrils and glucuronoxylan), resulting in reduced cell wall porosity and increased hydrophobicity (Mellorowicz et al. 2001; Ruel et al. 2006). Thus, the presence of xylan and lignin in the S- and L-layers of G-fibers would ensure mechanical support, while the absence of these components in the conventional G-layer would allow short-term water storage in plants subject to water deficit (Jönsson 1902; Carlquist 2012). In fact, the presence of G-fibers in plants that occur in environments that present water deficit, as well as the Cerrado, has been described in different species, with habit ranging from herbaceous (Paviani 1974; Piva et al. 2019) to arboreous (Marcati et al. 2001; Luchi 2004; Milanez et al. 2017). Cerrado is a seasonal, fire-prone ecosystem characterized by dystrophic and acidic soils with low nutrient availability, high levels of soluble aluminum (Coutinho 2002; Bueno et al. 2013). Under these conditions, the presence of non-lignified (hydrophilic) G-layer in G-fibers in the primary vegetative axis of *E. simplicifolium,* a frequent herbaceous species in the Cerrado, may perform several functions, such as short-term unfavorable water storage (Jönsson 1902; Carlquist 2012, 2014), aluminum accumulation (Milanez et al. 2017) and underground organ contraction

in water deficit and burned conditions, favoring the protection of these organs (Fisher 2008). In case the G-layer is partially lignified, therefore hydrophobic and rigid, the G-fibers would act as mechanical support to the aerial and/or underground organs, conferring resistance of these plants in adverse situations, such as drought, fire passage and herbivores.

Conclusion

The results of this study showed that the organization and distribution of G-fibers and G-layer composition vary between organs of the same individual. The presence of G-layer lignification, characterizing the occurrence of composed G-layer, enables new interpretations for the physiological role of G-fibers. Future studies with experimental approach may elucidate the factors that act in the formation of the L-layer in the G-fibers.

Acknowledgements Thanks go to the staff of the Institute Kazan Biochemistry and Biophysics and Profª Drª Tatyana A. Gorshkova for all their support.

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Table

Table 1 - Immunolabeling results for xylans and galactans. Caption: SCW: secondary cell wall (S-layer), TCW: tertiary cell wall (conventional G-layer), L: lignified lamella (L) of composed G-layer.

Organ analyzed	**LM10** (xylans)	**LM5** (galactans)
Midrib	SCW and L	TCW
Apex stem	SCW and L	TCW
Stem internode	SCW and L	TCW
Underground Organ	SCW and L	TCW

Figures

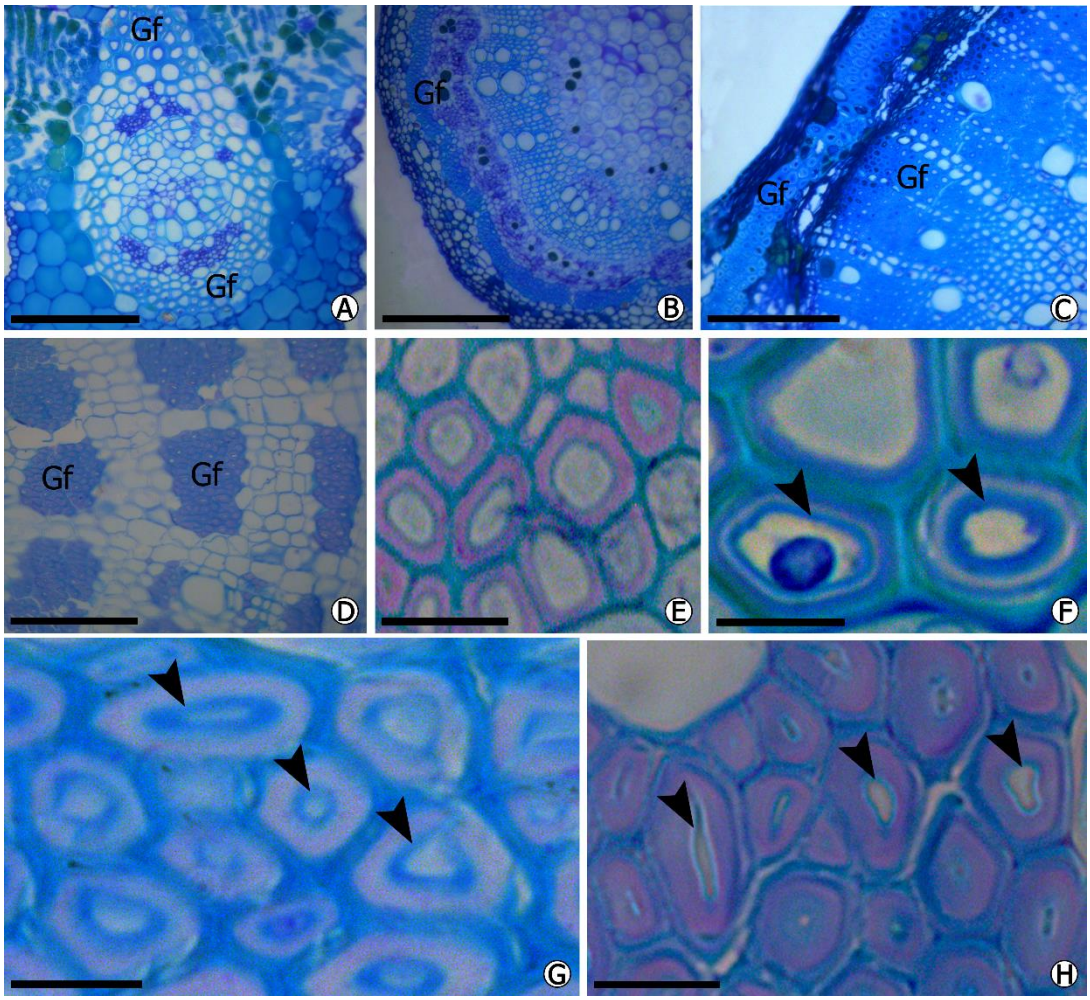

Figure 1 - Photomicrographs of the cross sections showing G-fibers in *Eriosema simplicifolium.* **a** Midrib with G-fibers (Gf) arranged in caps on the dorsal surface and small groups on the ventral surface around the phloem. **b** Primary growing stem with G-fibers (Gf) arranged in complete cylindrical strands around the phloem. **c** Secondary growth stem with G-fibers (Gf) in complete cylindrical cord around the phloem and in groups separated by parenchyma rays. **d** Secondary growth root with G-fibers (Gf) in small groups distributed in tangential bands separated by parenchyma rays. **e** Primary growth stem with G-fibers that have conventional G-layer. **f** Midrib with G-fibers that have composite G-layer (arrowhead). **g** Secondary growth stem with G-fibers that have composite G-layer (arrowhead). **h** Root with G-fibers that have no composite G-layer (arrowhead). Bars: **a, c** and **d** = 100µm; **b** = 200µm; **e** and **h** = 50µm; **f** and **g** = 20µm.

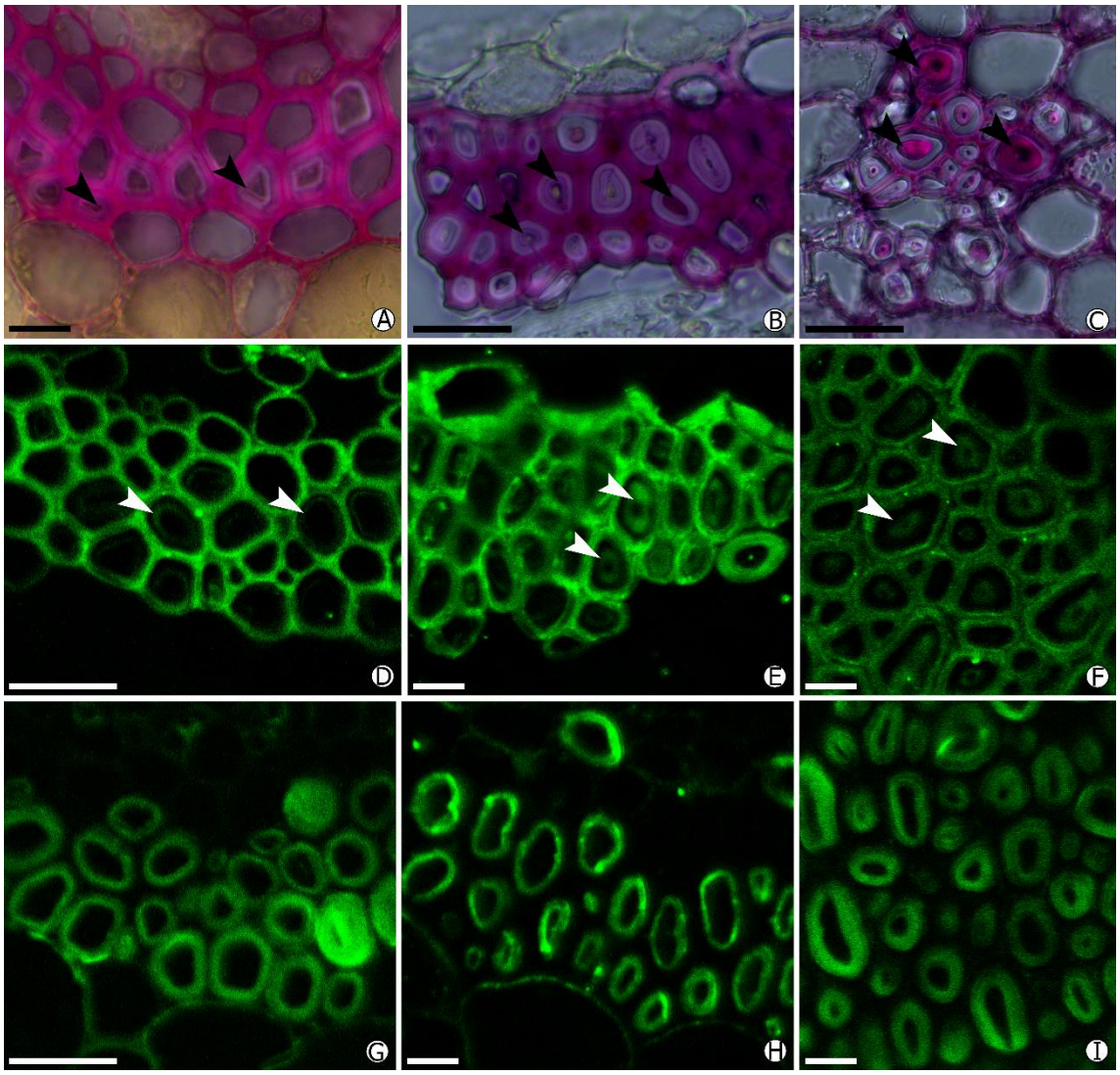

Figure 2 - Photomicrographs of cross sections showing G-fibers in *Eriosema simplicifolium* using phloroglucine acidic (**a-c**) and immunostaining with antibodies LM10 (**d-f**) and LM5 (**g-i**). **a** Midrib with G-fibers that have lignified composite G-layer (black arrowhead). **b** Secondary growth stem with G-fibers that have lignified composite G-layer (black arrowhead). **c** Root with G-fibers that have lignified composite G-layer (black arrowhead). **d** Midrib with G-fibers having G-layer composed of xylan (white arrowhead). **e** Secondary growth stem with G-fibers that have G-layer composed of xylan (white arrowhead). **f** Root with G-fibers that have G-layer composed with xylan (white arrowhead). **g** Midrib with G-fibers having G-layer with galactan. **h** Secondary growth stem with G-fibers that have G-layer with galactan. **i** Root with G-fibers that have G-layer with galactan. Bars: **a, d** and **g** = 20µm; **b** and **c** = 50µm; **e-f, h-i** = 10µm**.**

CONSIDERAÇÕES FINAIS

- As condições ambientais do Cerrado parecem atuar na diferenciação de fibras gelatinosas no eixo vegetativo de *Eriosema*, e a alta frequência de fibras gelatinosas nessas plantas pode ser um mecanismo adaptativo associado com armazenamento temporário de água, além de suporte mecânico.
- A presença de lignificação da camada-G, caracterizando a ocorrência de uma camada-G composta, possibilita novas interpretações para o papel fisiológico das fibras gelatinosas.
- A combinação de métodos histoquímico e imunohistoquímico abre novas perspectivas na compreensão da arquitetura e composição das paredes das fibras gelatinosas.
- Análises ultraestruturais das paredes das fibras gelatinosas sob o ponto de vista ontogenético são necessárias para compreender os processos de síntese, deposição e formação da camada-G composta.
- Estudos futuros com abordagem experimental podem elucidar os fatores que atuam na formação da camada-G composta nas fibras gelatinosas.