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UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus Botucatu



Diferenciação de laticíferos articulados em plantas de *Tabernaemontana catharinensis* (Apocynaceae)

YVE CANAVEZE

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

BOTUCATU-SP

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YVE CANAVEZE

PROF^A DR^A SILVIA RODRIGUES MACHADO
ORIENTADORA

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RESUMO – Neste estudo buscamos avaliar diferentes aspectos da diferenciação de laticíferos. O crescimento intrusivo (capacidade de crescer penetrando a lamela média de células adjacentes) e a ação indutora dos laticíferos (capacidade de induzir uma célula vizinha a adquirir características de laticíferos) são mecanismos de crescimento interessantes que podem ocorrer nos laticíferos, mas que são mal compreendidos. O etileno e o ácido jasmônico são hormônios vegetais liberados durante a herbivoria e podem afetar a diferenciação de estruturas secretoras relacionadas à defesa, como laticíferos e ductos resiníferos. Utilizando métodos convencionais em microscopia eletrônica de transmissão, para caracterização da ultraestrutura celular, e imunocitoquímicos, para a detecção de componentes de parede celular, exploramos o desenvolvimento de laticíferos articulados anastomosados com crescimento intrusivo e a ação indutora dos laticíferos no embrião maduro e na planta de *Tabernaemontana catharinensis* A.DC. (Apocynaceae). Além disso, pulverizamos soluções de etileno 2%, ácido jasmônico 0,1% ou água deionizada em plantas ($n = 33$) de *T. catharinensis* cultivadas em condições controladas e avaliamos a influência destes reguladores vegetais sobre a diferenciação dos laticíferos nos tecidos primários e floema secundário. Ultraestruturalmente, verificamos que o processo de incorporação de células ao sistema laticífero é distinto no embrião e na planta. Entretanto, em ambos os casos, o protoplasto da célula incorporada adquire características semelhantes à de laticíferos, e a incorporação é restrita às células em contato com laticíferos. Os tratamentos imunocitoquímicos revelaram que as paredes celulares dos laticíferos e

das células do meristema fundamental no embrião maduro marcaram de maneira semelhante para JIM7 e JIM5 (homogalaturonanos com grau de alta e baixa de metil-esterificação, respectivamente), LM6 ((1→5)- α -L-arabinanos) e BS400-2 (calose). Em porções indiferenciadas do ápice caulinar na planta, as paredes dos laticíferos marcaram mais intensamente para JIM5 e LM6 e exibiram fraca ou nenhuma marcação para LM5 ((1→4)- β -D-galactanos) e BS400-2 do que as células meristemáticas adjacentes. A detecção de epitopos glicanos no ápice caulinar da planta é compatível com crescimento intrusivo em laticíferos. Análises quantitativas revelaram que a diferenciação dos laticíferos originados do meristema fundamental (córtex e medula) é reduzida por etileno exógeno, enquanto o ácido jasmônico parece não influenciar a diferenciação dos laticíferos. A diferenciação dos laticíferos originados do procâmbio e do câmbio vascular parece não ser influenciada pelo etileno e ácido jasmônico exógenos. Assim, considerando as funções protetoras do látex, plantas tratadas com etileno exógeno podem ficar mais vulneráveis ao ataque de herbívoros e patógenos.

Palavras-chave: ácido jasmônico, Apocynaceae, imunocitoquímica, etileno, sistema laticífero, ultraestrutura

Canaveze, Y. **Differentiation of articulated laticifers in *Tabernaemontana catharinensis* (Apocynaceae)**. 2016. TESE DE DOUTORADO – INSTITUTO DE BIOCÊNCIAS, UNESP – UNIVERSIDADE ESTADUAL PAULISTA, CAMPUS DE BOTUCATU.

ABSTRACT – In this study, we evaluated different aspects of the laticifer differentiation. The intrusive growth (the capacity of grow penetrating the middle lamella that cements adjacent cells) and the inducing action of laticifers (the capacity of induced an adjacent cell to acquirer laticifer features) are exciting growth mechanisms that may occur in laticifers, but they are poorly understood. Ethylene and jasmonic acid are plant hormones released during the herbivory and may affect the differentiation of secretory structures related with plant defense, as laticifers and resin ducts. Using transmission electron microcopy conventional methods (for characterization of the cell ultrastructure), and immunocytochemistry (for detection of the cell wall compounds), we explored the development of the articulated anastomosing laticifers with intrusive growth and the induction action of laticifer in the mature embryo and plant of *Tabernaemontana catharinensis* A.DC. (Apocynaceae). Additionally, we sprayed solutions of the 2% ethephon, 0.1% jasmonic acid and deionized water in plants of *T. catharinensis* ($n = 33$) growing under controlled conditions and evaluated the influence of these plant regulators on laticifer differentiation in the primary tissues and secondary phloem. In the ultrastructure, we verified that the process of cell incorporation within the laticifer system is distinct in the embryo and plant. However, in both the cases the protoplast of the incorporated cells acquires similar features of laticifers, and the incorporation is restricted to those cells in contact with laticifers. The immunolocalization analyses revealed that walls of laticifers and ground meristem cells in the mature embryo have

similar labeled for JIM7 and JIM5 (homogacturonans with high and low degree of methyl-esterification, respectively), LM6 ((1→5)- α -L-arabinans) and BS400-2 (calose). In undifferentiated portions of the plant shoot apex, the laticifer walls show more intense labeling for JIM5 and LM6 and exhibit weak or no labeling for LM5 ((1→4)- β -D-galactans) and BS400-2 than the adjacent meristematic cells. The labeling of glycan epitopes in the shoot apex of plant is compatible with intrusive growth in laticifers. Quantitative analyzes revealed that the differentiation of laticifers from ground meristem (in cortex and pith) is reduced by exogenous ethylene, while jasmonic acid appears not to influence on the laticifer differentiation. Laticifer differentiation from procambium and vascular cambium seems be not influenced by ethylene or jasmonic acid. Thus, considering the protective latex functions, we suggest that the negative effect of exogenous ethylene on laticifer differentiation in *T. catharinensis* would make the plants most vulnerable to attack by herbivores and pathogens.

Key words: Apocynaceae, ethylene, immunocytochemistry, jasmonic acid, laticifer system, ultrastructure

Introdução

Laticíferos, estruturas secretoras internas portadoras de látex, formam um sistema que permeia vários tecidos e órgãos no corpo da planta e representam um potente sistema de defesa contra predadores tais como insetos e microorganismos e como selante de ferimentos (Fahn 1979, 1990, Evert 2006), além de beneficiarem as plantas ao isolarem substâncias químicas de defesa dos tecidos sensíveis (Farrell et al. 1991). O papel defensivo do látex tem sido suportado por muitas linhas de evidência, e alguns autores atribuíram o maior sucesso das plantas latescentes nos diversos ambientes, em relação às não latescentes, às funções protetoras do látex (Farrell et al. 1991, Agrawal & Konno 2009, Konno 2011).

Estruturalmente, os laticíferos são classificados em dois tipos: não-articulados e articulados (Esau 1953). Laticíferos não-articulados são formados por uma única célula isolada com crescimento indeterminado, diferenciando-se em estruturas tubulares que apresentam crescimento intrusivo, podendo ser ramificados ou desenvolverem-se em tubos mais ou menos em linha reta, sendo classificados como não-ramificados (Evert 2006). Laticíferos articulados são formados por fileiras de células alongadas, que se dispõem em série, podendo suas paredes terminais permanecer íntegras ou serem parcial ou totalmente dissolvidas (Evert 2006). Se houver conexões entre fileiras axiais distintas, os laticíferos são classificados como articulados anastomosados, em não ocorrendo tais conexões, os laticíferos são classificados como articulados não-anastomosados (Evert 2006).

Em laticíferos articulados, a incorporação de células parenquimáticas (em tecidos diferenciados) ou de células do meristema fundamental (em tecidos indiferenciados) ao sistema laticífero tem sido relatada para diferentes espécies (Milanez 1959, 1977, Lopes et al. 2009, Canaveze & Machado *in press*). A ramificação horizontal em laticíferos do

embrião maduro de *Nerium oleander* (Apocynaceae), parece ser resultado da ação indutora do laticífero sobre uma célula meristemática vizinha; o laticífero emite uma projeção lateral fina que penetra no citoplasma da célula adjacente e, posteriormente, essa célula se diferencia apresentando características de laticíferos (Milanez 1977). A ação indutora do laticífero ainda não é clara e merece ser estudada.

Laticíferos articulados e não articulados podem se ramificar em regiões meristemáticas ou de alongamento por crescimento intrusivo através de projeções apicais ou laterais (Rudall 1987, Canaveze and Machado *in press*). Este tipo de crescimento tem sido estudado com maior detalhe em laticíferos não articulados (Serpe et al. 2001, 2002, 2004) e requer alterações e rearranjos dos componentes de parede celular, além da quebra de conexões simplásticas (Mahlberg 1993, Serpe et al. 2002). Métodos imunocitoquímicos para a localização de componentes de parede celular podem auxiliar na compreensão da dinâmica da parede durante o crescimento intrusivo em laticíferos (Serpe et al. 2001, 2002, 2004).

Diversas linhas de evidência demonstram que a produção de látex é afetada por condições bióticas e abióticas, dentre elas a herbivoria (Agrawal & Konno 2009). As injúrias causadas na planta durante a herbivoria estimulam a produção de hormônios vegetais que desencadeiam diferentes respostas fisiológicas nas plantas: o etileno é o hormônio liberado em função do estresse mecânico e o ácido jasmônico, em função ao estresse químico (Taiz & Zeiger 2006).

Nesse sentido, alguns estudos têm buscado compreender a atuação dos hormônios/reguladores vegetais na formação de estruturas secretoras relacionadas à defesa das plantas, como ductos e laticíferos. Etileno (Yamamoto & Kozlowski 1987, Fahn 1988, Hudgins & Franceschi 2004) e metil jasmonato (Hudgins & Franceschi 2004)

exógenos induzem a formação de ductos resiníferos traumáticos em diferentes espécies, especialmente coníferas.

Em cultivos comerciais de *Hevea brasiliensis* (Euphorbiaceae), o ethephon, um agente liberador de etileno, é comumente aplicado para estimular o fluxo de látex (Hao & Hu 2000). Entretanto, o etileno não causa alterações quantitativas observáveis nos laticíferos de indivíduos de *H. brasiliensis* em que não há coleta de látex (Hao & Hu 1984). A aplicação de ácido jasmônico e ácido linolênico em plantas de *H. brasiliensis* promovem o aumento do número de laticíferos no córtex e no floema secundário (Hao & Hu 2000). O ácido linolênico é precursor do ácido jasmônico e esses hormônios são associados à defesa contra patógenos e ataque de insetos (Hao & Hu 2000). Nesse mesmo estudo, a aplicação de ácido abscísico, etileno e ácido salicílico não promovem efeito detectável na diferenciação dos laticíferos (Hao & Hu 2000). Assim, a influência dos reguladores vegetais na diferenciação de laticíferos merece ser melhor investigada.

Tabernaemontana catharinensis A.DC. (Apocynaceae) tem uma ampla distribuição geográfica que se estende a diferentes domínios fitogeográficos do Brasil (Koch et al. 2015). Esta espécie é abundante em áreas com forte alteração antrópica, além de ocorrer em pastagens e culturas (Pott et al. 2006). O extrato desta planta exhibe atividades tripanocida (Pereira et al. 1999), leishmanicida (Soares et al. 2007), anti-tumoral (Almeida et al. 2004) e anti-ofídica (Batina et al. 2000, Almeida et al. 2004, Veronese et al. 2005, Gomes et al. 2010). Alcalóides indólicos com diferentes propriedades farmacológicas e aplicações em medicina popular também foram registrados *T. catharinensis* (Pereira et al. 2004, Gonçalves 2011).

T. catharinensis possui laticíferos articulados anastomosados com crescimento intrusivo nos corpos primário e secundário da planta e seu desenvolvimento envolve

fusão de protoplastos, adição de células e crescimento intrusivo (Canaveze & Machado *in press*).

Neste estudo buscamos compreender aspectos importantes da diferenciação de laticíferos articulados. Utilizando métodos convencionais em microscopia eletrônica de transmissão, para caracterização da ultraestrutura celular, e imunocitoquímicos, para a detecção de componentes de parede celular, exploramos o desenvolvimento de laticíferos articulados anastomosados com crescimento intrusivo e a ação indutora dos laticíferos em embrião maduro e planta de *T. catharinensis*. Outro aspecto da diferenciação dos laticíferos que avaliamos foi a influência do etileno e ácido jasmônico sobre a diferenciação dos laticíferos nos tecidos primários e floema secundário, utilizando plantas de *T. catharinensis* cultivadas em condições controladas.

Nosso estudo, que leva em conta diferentes aspectos referentes à diferenciação de laticíferos, pode contribuir para programas de melhoramento da produção e da coleta de látex, porque além das diferentes funções ecológicas já apresentadas, alguns componentes do látex também apresentam importância econômica relevante (Agrawal & Konno 2009).

Revisão Bibliográfica

Estrutura e ultraestrutura do desenvolvimento de laticíferos

Laticíferas são estruturas secretoras que consistem de células isoladas ou fileiras de células especializadas que contém látex, formando um sistema que permeia vários tecidos e órgãos no corpo da planta (Evert 2006). Estruturalmente, os laticíferos são classificados em dois tipos: articulados e não articulados (De Bary 1884, Esau 1953, Fahn 1979).

Laticíferos não articulados têm origem exclusivamente nas iniciais laticíferas no embrião, são formados por uma única célula isolada com crescimento indeterminado, diferenciando-se em estruturas tubulares que apresentam crescimento intrusivo, podendo ser ramificados ou desenvolverem-se em tubos mais ou menos em linha reta, sendo classificados como não ramificados (Evert 2006).

Laticíferos articulados são formados por fileiras de células alongadas, que se dispõem em série, podendo suas paredes terminais permanecer íntegras ou serem parcial ou totalmente dissolvidas (Evert 2006). Se houver conexões entre fileiras axiais distintas, os laticíferos são classificados como articulados anastomosados, em não ocorrendo tais conexões, os laticíferos são classificados como articulados não-anastomosados (Evert 2006). Laticíferos articulados se originam da fusão de protoplastos, derivados dos meristemas primários ou secundários, após a dissolução parcial ou total das paredes transversais, podendo estar ou não presente no embrião (Milanez 1977).

A estrutura do sistema laticífero pode influenciar na vulnerabilidade das plantas quanto ao ataque de herbívoros. Folhas com laticíferos articulados são mais bem protegidas contra herbívoros mandibulados em relação às que possuem laticíferos não-articulados, porque um único corte no laticífero não articulado da nervura principal

interrompe todo o fluxo do látex acima do local lesado; no caso do laticífero articulado anastomosado, mesmo que o inseto corte várias nervuras, existem outras rotas que permitem a continuidade do fluxo (Agrawal & Konno 2009, Konno 2011).

A estrutura do sistema laticífero também é uma característica importante e que, de certa forma, permite que a planta seja utilizada comercialmente em grande escala e com grande produtividade de látex. Apesar de a borracha natural ser produzida por mais de 2.500 espécies vegetais, *Hevea brasiliensis* (Euphorbiaceae) permanece sendo a única fonte economicamente importante deste produto (Evert 2006). Além é claro das propriedades de seu látex, *Hevea brasiliensis* possui laticíferos articulados anastomosados no floema secundário, principalmente na região do floema colapsado (Evert 2006), por isso durante a coleta do látex, os laticíferos interconectados exudam grande quantidade de látex.

Os laticíferos variam amplamente quanto à origem. Tais estruturas secretoras podem se originar a partir do meristema fundamental e/ou procâmbio (Fahn 1979, Matile 1987), do câmbio, felogênio ou de células parenquimáticas (Milanez 1959, 1977, Lopes et al. 2009). Os relatos apontam que a fase meristemática é muito breve e, enquanto a maioria dos tecidos ainda é meristemática, os laticíferos já estão diferenciados e em fase secretora (Fahn 1979, Matile 1987, Demarco et al. 2006).

Nos laticíferos, o látex preenche tais células desde suas porções mais jovens, sendo composto pelo próprio protoplasto (Matile 1987). Do ponto de vista citológico, no início do desenvolvimento dos laticíferos, não articulados e articulados, o citoplasma é denso, pode apresentar um ou mais núcleos proeminentes, inúmeras mitocôndrias, retículo endoplasmático rugoso, corpos de Golgi, ribossomos e plastídios (Evert 2006). Durante a diferenciação, ocorre proliferação de vesículas e pequenos vacúolos, que podem ser originados da dilatação de porções do retículo endoplasmático (Nessler &

Mahlberg 1977, Wilson & Mahlberg 1978, 1980, Cai et al. 2009). Inicia-se também o processo de autofagia celular que pode culminar num protoplasto com citoplasma periférico e amplo vacúolo central, onde é possível observar inúmeras partículas de látex (Fahn 1979). Esta conformação é frequentemente observada, tanto em laticíferos articulados quanto em não-articulados. Entretanto a autofagia celular pode ser mais intensa e culminar na formação de um protoplasto preenchido com vesículas e partículas de látex, como relatado para algumas espécies com laticíferos articulados (Evert 2006).

A origem dos núcleos difere entre os tipos de laticíferos. Nos laticíferos não articulados, a célula se alonga indefinidamente e novos núcleos são formados por cariocinese desacompanhada de citocinese, formando uma estrutura cenocítica (Milanez 1977), sendo registradas ondas mitóticas (Mahlberg 1966). Enquanto nos laticíferos articulados, continuamente são acrescentadas novas células e núcleos de diferentes células integram o laticífero. Em alguns laticíferos articulados a degradação entre paredes terminais e laterais entre laticíferos é precoce, completa e não deixa indícios das paredes; nestas condições, forma-se uma estrutura sincicial que pode ser equivocadamente considerada como uma estrutura cenocítica (Milanez 1952, 1977). A degeneração dos núcleos foi registrada para os dois tipos de laticíferos (Milanez 1952, Da Cunha et al. 1998).

Normalmente, o conteúdo dos laticíferos está sob alto turgor (Hagel et al. 2008, Dusotoit-Coucaud et al. 2009, Onoyovwe et al. 2013). Analisando distintos métodos de fixação e processamento, Condon & Fineran (1989) verificaram que o formato estrelado observado em laticíferos articulados de *Calystegia silvatica* (Convolvulaceae), possivelmente, é um artefato causado durante a coleta pelo colapso parcial do conteúdo das células em virtude da diminuição da pressão de turgescência, acompanhado pela expansão das células subsidiárias. Nos laticíferos articulados em *Papaver somniferum*

(Papaveraceae) também há relato de perda de turgor celular durante a fixação (Onoyovwe et al. 2013).

Mais frequentemente, os laticíferos possuem paredes primárias não lignificadas com espessura variável (Fahn 1979, Serpe et al. 2001, Demarco et al. 2006, Demarco & Castro 2008, Lopes et al. 2009, Demarco 2015). No entanto, há registros da presença de suberina (Fineran et al. 1988) e lignina (Rudall 1994) na parede de laticíferos.

Plasmodesmos raramente são encontrados em paredes comuns entre laticíferos e células adjacentes (Evert 2006). Faÿ et al. (1989), utilizando técnicas em microscopia de fluorescência com coloração de azul de anilina e microscopias eletrônicas de transmissão e varredura, verificaram que plasmodesmos numerosos ocorrem entre as células do parênquima radial e axial no floema secundário de *Hevea brasiliensis*, mas são raros ou ausentes em parede comuns entre laticíferos e células do parênquima. Portanto, os metabólitos que entram devem penetrar via aploplasto e devem ser transportados ativamente através da membrana plasmática do laticífero (Faÿ et al. 1989). Por exemplo, a sacarose é o precursor de borracha natural (*cis*-poliisopreno); assim a importação de sacarose para os laticíferos em *Hevea brasiliensis* (seringueira) é essencial para a produção de borracha (Dusotoit-Coucaud et al. 2009). Uma vez que as conexões simplásticas são raras ou ausentes há a necessidade de transportadores de membrana para sacarose. Indo mais profundamente nesta questão, é usual em culturas de seringueira a aplicação de etileno para estimular a produção de látex (Hao & Hu 2000, Dusotoit-Coucaud et al. 2009). O etileno não aumenta o número de laticíferos em seringueira (Hao & Hu 2000), portanto, o aumento de produção do látex deve ter outro motivo. Pensando em todos estes aspectos, Dusotoit-Coucaud et al. (2009) estudaram a expressão de transportadores de sacarose na membrana celular dos laticíferos em seringueira. Os autores verificaram que o etileno recruta os transportadores HbSUT1A e HbSUT2A

(isoformas de transportadores de sacarose) na membrana plasmática dos laticíferos. Estes transportadores importam sacarose para o citoplasma do laticífero com duas possíveis consequências que contribuem para o aumento da produção de látex: maior produção de borracha (maior rentabilidade do látex) e, juntamente com polióis e potássio, a sacarose contribui para aumentar a pressão de turgor (importante para o fluxo do látex durante a coleta) (Dusotoit-Coucaud et al. 2009).

O crescimento intrusivo é uma característica dos laticíferos que merece maiores investigações. Crescimento intrusivo é comumente associado à maneira de crescimento e ramificação de laticíferos não-articulados (Fahn 1979, Evert 2006), mas já foi reportado para laticíferos articulados para alguns gêneros de Euphorbiaceae como *Hevea*, *Manihot* e *Cnidoscolus* (Rudall 1987). Durante o crescimento intrusivo espera-se que ocorram alterações na parede celular, como desarranjo de componentes da parede celular e quebra de ligações via plasmodesmos entre as células meristemáticas (Wilson et al. 1976, Mahlberg 1993). A penetração dos laticíferos deve ser um balanço entre os processos de desarranjo e síntese de parede celular, logo que não são observados danos nas paredes dos laticíferos ou das células meristemáticas ao redor (Serpe et al. 2002). Neste sentido, os estudos de Serpe et al. (2001, 2002, 2004) merecem destaque.

Utilizando técnicas histoquímicas e imunocitoquímicas para localização de epitopos pécticos e calose em ápices caulinares de *Asclepias speciosa* (Apocynaceae), que possui laticíferos não articulados, Serpe et al. (2001, 2002) avaliaram as consequências do crescimento intrusivo na parede das células meristemáticas em contato com laticíferos. A penetração dos laticíferos causa alteração na parede celular das células meristemáticas adjacentes, mas não induz resposta de defesa, como por exemplo, acúmulo de calose (Serpe et al. 2002) ou de compostos fenólicos (Serpe et al. 2001) nas paredes. A alteração mais evidente é falta de calose nas paredes de contato laticífero-

célula meristemática. A ausência desse polissacarídeo indica o seu desarranjo durante a penetração do laticífero e pode facilitar a separação das células meristemáticas (Serpe et al. 2002). A lamela média é mais ampla entre a parede dos laticíferos e das células meristemáticas adjacentes e é rica em homogalacturonanos (HGs), reconhecidos pelo anticorpo monoclonal JIM5; o crescimento dos laticíferos é acompanhado pela deposição de HGs e por modificação destes polissacarídeos por pectina metilesterases (Serpe et al. 2002). Além de a lamela média ser rica em HGs, há diferenças na distribuição de epitopos de rhamnogalacturonanos I (RGI). (1→4)-β-galactanos, reconhecido pelo anticorpo monoclonal LM5, não marcaram as paredes celulares comuns entre laticíferos e células meristemáticas, enquanto (1→5)-α-L-arabinanos, reconhecido pelo anticorpo monoclonal LM6, é abundante nestas paredes. A lamela média rica em pectinas pode facilitar o transporte de água e nutrientes pela parede celular e pode atuar como lubrificante, diminuindo o atrito entre as paredes durante a penetração dos laticíferos (Serpe et al. 2002).

Em estudo posterior com os mesmos anticorpos, mas em laticíferos não articulados de *Euphorbia heterophylla* (Euphorbiaceae), Serpe et al. (2004) não observaram danos nas paredes das células meristemáticas adjacentes à laticíferos e verificaram que a maior diferença entre as paredes celulares de laticíferos e células meristemáticas é a ausência de epitopos (1→4)-β-galactanos, reconhecido por LM5, na parede dos laticíferos. Calose também não foi detectada na parede em comum de laticíferos e células meristemáticas. Estas duas características são compartilhadas com os laticíferos não articulados de *Asclepias speciosa*, possivelmente os laticíferos destas espécies são estruturas análogas resultado de convergência evolutiva (Serpe et al. 2004). Os autores concluíram que se ocorrem desarranjos de polissacarídeos durante a

penetração dos laticíferos por entre células meristemáticas, estes são espacial e temporalmente confinados a pequenas regiões da parede celular (Serpe et al. 2004).

A ação indutora dos laticíferos é outra característica dos laticíferos, somente dos articulados, que merece estudos adicionais. Segundo Milanez (1977), a formação de novos ramos horizontais nos laticíferos do embrião maduro de *Nerium oleander* (Apocynaceae), geralmente, parece ser resultado de ação indutora do laticífero sobre uma célula vizinha do meristema fundamental; o laticífero emite uma projeção lateral fina que penetra no citoplasma da célula vizinha e, posteriormente, esta célula se diferencia apresentando características de laticíferos. As células do meristema fundamental apresentam como reserva grãos protéicos e gotas lipídicas, nos laticíferos já diferenciados estas substâncias não são observadas, mas há laticíferos que apresentam estas substâncias indicando sua recente incorporação (Milanez 1977). Em *Cryptostegia grandiflora* (Apocynaceae) a degradação dos grânulos de reserva em algumas células e a ausência destes grânulos nos laticíferos diferenciados também foi associada à incorporação de células ao sistema laticífero (Milanez 1959).

Os estudos com laticíferos têm sido especialmente focados na sua identificação e caracterização morfológica em inúmeras espécies (Hagel et al. 2008). Ao longo das últimas décadas, os laticíferos vêm sendo estudados sob os aspectos bioquímicos, ecológicos e consequências evolutivas do látex (Agrawal & Konno 2009, Konno 2011).

Em síntese, estudos que desvendem os mecanismos morfológicos de crescimento intrusivo, ação indutora dos laticíferos, capacidade de coagulação do látex, substâncias promotoras de diferenciação, entre outros, são escassos e restritos a poucas espécies de interesse econômico. Outros aspectos como, o tempo de vida dos laticíferos, se tais estruturas senescem e são substituídas durante o desenvolvimento da planta sequer são discutidos. Mesmo com o avanço nos métodos de análise, por exemplo, uma variedade

de anticorpos específicos para componentes da parede celular, e equipamentos mais sofisticados, como microscópio confocal e tomógrafo, que permitiriam leitura tridimensional dos laticíferos, ainda faltam estudos que aprofundem o conhecimento da estrutura e funcionamento destas estruturas secretoras.

Os resultados obtidos durante a execução deste projeto de doutorado foram redigidos na forma de artigos segundo as normas da revista selecionada para publicação seguindo a P O R T A R I A Nº 02/2011-SPG/IBB/UNESP.

Capítulo I - Change in glycans epitopes and cellular dynamic on the incorporation of cells and intrusive growth in articulated laticifers of *Tabernaemontana catharinensis* A.DC. (Apocynaceae)

Revista: **PlosOne**

Capítulo II - Influence of exogenous ethylene and jasmonic acid on the laticifers differentiation: evidence obtained from *Tabernaemontana catharinensis* (Apocynaceae) plants

Revista: **Botany**

Change in glycans epitopes and cellular dynamic on the incorporation of cells and intrusive growth in articulated laticifers of *Tabernaemontana catharinensis*

A.DC. (Apocynaceae)

Abstract

Laticifers, cells that contain latex, exhibit interesting growth mechanisms in plants; including intrusive growth (i.e. the capacity of grow penetrating the middle lamella that cements adjacent cells) or inducing adjacent cells to acquire laticifer features, as accepted for some authors. Using transmission electron microcopy and immunocytochemical methods, we aimed to elucidate the differentiation of a complex structure, which involve protoplast fusion, the addition of cells and intrusive growth, as the case of the laticifer system in *Tabernaemontana catharinensis* A.DC. (Apocynaceae), comparing mature embryo and plant. Laticifers are axially elongated, irregular in outline and form projections where there is contact with two or more cells. The process of cell incorporation within the laticifer system is distinct in the embryo and plant. In both the cases the protoplast of the incorporated cells acquires similar features of laticifers, and the incorporation is restricted to those cells in contact with laticifers. Laticifer cell walls in embryo labeled for JIM7 and JIM5 (homogacturonans with high and low degree of methyl-esterification, respectively), LM6 (arabinans) and BS400-2 (calose). In undifferentiated portions of the shoot apex, the laticifer walls show more intense labeling for JIM5 and LM6 and exhibit weak or no labeling for LM5 (galactans) and BS400-2 than the adjacent meristematic cells. In the differentiated portions of the shoot apex, laticifers labeling is similar to the adjacent parenchyma cells. Glycans epitopes of the laticifer walls differ in the embryo and plant and in the different regions of a same laticifer. Our data on laticifer wall glycans epitopes are compatible with intrusive growth. Although the incorporated cells exhibit

subcellular features of laticifers, we were not able to verify changes in glycan epitopes wall of these cells.

Introduction

Laticifers are grouped into two types: articulated and nonarticulated. This classification is based on their ontogenesis and structure, and both types vary in the degree of complexity of their structure [1]. Articulated laticifers consist of longitudinal rows of cells in which the walls separating the individual cells either remain intact, become perforated, or are completely removed [2,3]. They may form lateral connections with similar cell chains, all united into a netlike structure or reticulum, constituting an anastomosing system [1].

On a general way, the differentiation of the articulated laticifers involves either perforation or resorption of the cell wall and autophagy of protoplast [1]. Protoplast changes culminate in a large vacuole filled with a variety of substances, including latex particles [4] or the vacuolar membrane disappears and the lumen of the cell becomes filled with vesicles and latex particles and only the plasma membrane remains intact and functional [5]. Detailed cytological analyses are needed to understand the cell walls and protoplast changing on a developmental point a view.

The incorporation of meristematic or parenchyma cells to laticifer system during the development of articulated laticifers has been reported for some authors [6,7,8,9]. The inducing action of laticifers driving the incorporation of cells in the laticifer system was highlighted by Milanez [7]. However, this issue is still unclear and deserves to be researched.

In addition, articulated laticifers can elongate intrusively by tip or lateral projections, and ramify throughout the meristematic and elongating regions [9,10].

Regarding to intrusive growth the cell wall changes include disassembly or rearrangement of cell wall compounds and breaking of plasmodesmatal connections among laticifers and adjacent cells [11,12].

For understanding the dynamics of the cell wall during developmental process of laticifers immunocytochemical methods have shown promise [12,13,14]. Laticifer walls are predominantly primary and rich in pectins [11], and the polysaccharides composition vary among species [12,14]. Pectins are a diverse class of galacturonic acid-rich in polysaccharides and are classified into three main domains (homogacturonan – HG, rhamnogacturonan I – RG-I and rhamnogacturonan II – RG-II) [15,16]. Cell walls of laticifers and adjacent meristematic cells of *Asclepias speciosa* (Apocynaceae) differ in the expression of pectin epitopes, being (1→5)- α -arabinan epitope more abundant in laticifers than other cells, while the opposite was observed for a (1→4)- β -galactan epitope [12, 13]. In this species, the middle lamella between laticifers and adjacent cells was rich in HGs with a low degree of esterification, being such features associated with intrusive growth of laticifer [12]. In addition to pectins, other polysaccharides are present in laticifer cell walls, and callose can be low abundant or absence in laticifers and adjacent cells walls [12]. During intrusive growth of laticifers partial disassembly or rearrangement of polysaccharides molecules may also occur [12]. It is remarkable that all these studies were performed in nonarticulated laticifers. To best of our knowledge, there is no information on articulated laticifers.

In a previous study in the mature embryo, seedling and plant of the *Tabernaemontana catharinensis* A.DC. (Apocynaceae), we verified that this species has articulated anastomosing laticifers with intrusive growth [9]. Based on polysaccharides changing in nonarticulated laticifers during intrusive growth [12, 13,

14], we hypothesized that glycans distribution in the cell walls in articulated laticifers might support the same intrusive process as in nonarticulated laticifers.

We used conventional transmission electron microscopy and immunocytochemical methods to exploring the cell wall changes during the development of articulated anastomising laticifers with intrusive growth, as well as, to evaluate the inducing action of laticifers in *T. catharinensis*. More specifically, we explore the protoplast and wall changes of the laticifers and adjacent cells looking for the cell incorporation and the intrusive growth processes comparing mature embryo and plant.

Material and methods

Plant material

We studied mature embryo and plants of *T. catharinensis*. To obtain to these phases, we collected seeds from mature fruits of *T. catharinensis* plants growing in areas of cerrado (savanna-like vegetation) localized in in the municipalities of Botucatu (22°42'21.4''S and 48°18'23.7''W; 22°51'16.8''S and 48°25'53.2''W) and Pratânia (22°48'48.8''S and 48°44'33.7''W), in the state of São Paulo, Brazil during 2010, 2011, 2013 and 2014 (BOTU Herbarium, Vouchers Nos. 27625–27627). We removed mature embryos from soaked seeds and we cultivated 80-day-old plants under controlled conditions [17].

We collected and processed samples from the cotyledonary nodal region of mature embryo and the shoot apex and three phytomers below the shoot tip of 80-day-old plants.

Conventional methods for transmission electron microscopy (TEM)

We fixed the samples for 24 h at 5 °C in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) then post-fixed in a 1% osmium tetroxide (OsO₄) aqueous solution for 2 h at 25 °C in the same buffer. We dehydrated the samples in acetone series and embedded in Araldite resin (Araldite 502, Electron microscopy sciences, Hatfield, USA). We stained the ultrathin sections with uranyl acetate and lead citrate [18] and then examined them with a TEM (FEI TecnaiTM) at 80 kV.

Immunocytochemistry

1. Fixation, dehydration, embedding and sectioning

We fixed the samples for 48 h in 1% [v/v] glutaraldehyde and 4% [w/v] commercial formaldehyde in 0.2 M phosphate buffer (pH 7.2) [19]. Samples to analyze in TEM were post-fixed in a 1% [v/v] osmium tetroxide (OsO₄) aqueous solution for 1 h at 25 °C in the same buffer.

For experiments involving localization of callose, we incubated some samples with the inhibitor of callose synthesis 2-deoxy-D-glucose (DDG) in 10⁻⁴M for 1 h at 25 °C prior to fixation [12, 20, 21]. Subsequently, samples were fixed as described previously.

We dehydrated all the samples in alcohol series, embedded in LR White resin (Hard Grade - London Resin Company) and polymerized in gelatin capsules at 50 °C for 24 h. Semi-thin (500 nm) sections of samples with glass knives in microtome (Leica RM 2165) were made and collected on glass slides coated with poly-L-lysine (Sigma Aldrich). Testimonial sections were stained with Toluidine Blue pH 4.7 [22]. Ultrathin (80nm) sections of samples post-fixed in OsO₄ with a Diatome diamond knife were made and collected in 200 mesh gold grids.

2. Immunofluorescence microscopy

We used a range of monoclonal antibodies (Mabs) directed against homogalacturonan (HG), such as JIM5, JIM7 [23]; and rhamnogalacturonan I (RGI), such as LM5 [24], LM6 [25]. JIM5 and JIM7 recognize homogalacturonan (HG) epitopes with low and high degrees of methyl-esterification, respectively [23]. LM5 and LM6 recognize (1→4)-β-D-galactans [24] and (1→5)-α-L-arabinans [25], respectively. These antibodies were kindly provided by Prof. Paul Knox (Centre for Plant Sciences, University of Leeds, UK). Slides were incubated in a 1:10 dilution of primary antibody in MP/PBS for 2 h. After washing with PBS, slides were incubated in a 1:100 dilution of anti-rat-IgG (whole molecule) linked to fluorescein isothiocyanate (FITC, Sigma-Aldrich) in MP/PBS for 2 h. Slides were mounted in 0.1% [w/v] para-phenylenediamine (PPD).

We also used MAbs to detect (1→3)-β-D-glucans, including callose (BS400-2) [26], and (1→3),(1→4)-β-D-glucans (also known as or mixed-linkage glucan or MLG) (BS400-3) [27], which were purchased from Biosupplies Australia (Parkville, Australia) (Table 1). Sections were incubated in a 1:200 dilution of BS400-2 and 1:50 dilution of BS400-3 in MP/PBS for 2 h. After washing, these sections were incubated in a 1:100 dilution of goat anti-mouse-IgG (whole molecule) linked to FITC (Sigma-Aldrich) in MP/ PBS for 1 h.

Furthermore, a pre-treatment for pectic homogalacturonan (HG) removal with Na₂CO₃ 0.1 M, pH 11.4/pectate lyase (from *Aspergillus* sp., Megazyme) in Tris-HCl buffer, pH 8.0 was performed prior to labeling with LM5 and LM6 because pectic HG may mask sets of hemicellulose and other pectin epitopes [28, 29, 30].

After incubation with secondary antibody, we washed the sections with PBS and incubated with 0.01 % Calcofluor white (Fluorescent Brightener 28, Sigma Aldrich) for 5 min. Slides were mounted with an anti-fade solution consisting of 0.1%

PPD (Sigma-Aldrich) in 1:9 10 mM sodium phosphate buffer: 0.15 M NaCl and 90 % glycerol. All the steps were performed at room temperature (~ 25 °C).

Observations were made with a microscope equipped with epifluorescence (excitation filter 450–490 nm; microscope Leica DMR). Controls were made by omitting incubation with the primary antibody. The material was also checked for autofluorescence.

Serial sections of shoot apex of plants ($n = 3$) and cotyledonary node of embryos ($n = 3$) were used for all treatments. We compared samples of shoot apex ($n = 3$) and cotyledonary node ($n = 3$) prior fixed with DDG with samples non prior fixed to control BS400-2 antibody (callose).

3. Immunogold labeling for MET

Ultrathin sections (70 nm) on gold grids were dehydrated in PBS and MP/PBS. These sections were incubated with the MAbs as mentioned for "*Immunofluorescence microscopy*". After rinsing with PBS, sections were incubated with the appropriate secondary antibodies conjugated to 10-nm colloidal gold.

Binding of JIM5, JIM7, LM5 and LM6 to the sections was detected using goat anti-rat IgG (*whole molecule*)-Gold (Sigma Aldrich). Prior to labelling with LM5 and LM6, we performed incubation with pectate lyase in some sections (as described in "*Immunofluorescence microscopy*"). BS400-2 and BS400-3 were detected using a goat anti-mouse IgG (*whole molecule*)-Gold (Sigma Aldrich). To avoid callose epitope deposited by injury, sections labelling with BS400-2 were pre-fixed with DDG.

We applied secondary antibodies at a 1:20 dilution in MP/PBS for 2 h. Subsequently, the sections were washed in PBS and then in distilled water. The

incubations with primary antibody and both antibodies were suppressed in some sections. All the steps were performed at room temperature (~ 25 °C).

We examined the material with a TEM (FEI TecnaiTM) at 80 kV.

Results

Laticifers of *T. catharinensis* mature embryos and 80-days-old plants exhibited distinct characteristics related to the subcellular organization of protoplast and cell walls polysaccharides composition; thus, the results are showed separately for mature embryo and plant.

Laticifers ultrastructure

Mature embryo (Figs. 1, 2)

Laticifers are axially elongated (Fig 1a, b) and they may be irregular in outline and have projections where there is contact with two or more cells (Fig 1b). The cell walls have loose structure, a striate texture (Fig 1c), and may exhibit plasmodesmata (Fig 1d). Enlarged plasmodesmata channels (Fig 1e) and pores (Fig 1f) connecting the laticifer to an adjacent cell are observed. In the protoplast of the meristematic cells, just in the pore region, there are signs of degradation of the reserve content (Fig 2a).

The laticifer protoplast has variable content and low organelles definition (Figs. 1, 2). Laticifers have reduced cytoplasm interspersed with abundant lipid and protein bodies, but these ones with smaller dimensions when compared to the adjacent cells of the ground meristem (Fig 1a). During the laticifer development, signs of degradation of the reserve material can be noticed (Figs. 1a, 2a). The region of the laticifers protoplast formerly occupied with reserves becomes filled with electron-dense flocculated material dispersed in a finely granular medium (Fig 2a). There are

laticifers with electron-dense strands of rough endoplasmic reticulum (Fig 2b), and small vacuoles (Fig 2b, c). Eventually, mitochondria (Fig 2c) are observed.

Elongated cells irregular in outline and tip intrusive growth (Fig. 2d) occur in the embryo. These cells have intermediate features between the laticifers and the ground meristem cells (Fig 2d), exhibiting Golgi bodies with adjacent vesicles, and electron-opaque content, previously identified as lipids, dispersed in the protoplast (Fig 2d).

Shoot apex of plant (Figs. 3, 4)

Laticifers with distinct ultrastructural features are present in the shoot apex of plant (Fig 3a). The laticifers are distinguished from surrounding cells because they are elongated, irregular in outline and exhibit dense, loose cell walls and swollen middle lamella in regions where they are in contact with two or more cells (Fig 3a, b). The laticifer wall is thicker in the contact region with two or more cells (Fig 3c). Occasionally, obstructed plasmodesmata are observed as unusual electron-dense fibrillar arrangements within the common walls between laticifer and adjacent cell (Figure 3d), or peculiar electron-dense swellings within the adjacent wall (Fig 3e). Electron-lucent materials were observed immersed in the laticifers walls (Fig 3d); these materials (probably lipids) appear to be produced by plastids (Fig 3c inset) of the adjacent parenchyma cells and carried to the cell wall. End and lateral discontinuous walls occur between laticifers (Fig 3f).

The laticifer tips intrude among the adjacent cells forming an elongated and thin projection (Fig. 3g, h). Cortical microtubules (i.e. microtubules located in the peripheral cytoplasm just inside the plasma membrane) are observed in the meristematic cells in contact with the laticifer projection (Fig 3i). Microtubules are

oriented more or less perpendicular to the direction of elongation of the laticifer projection (Fig 3i).

Images suggesting the incorporation of meristematic cells to the laticifer system were frequently observed (Fig 4a-e). It is possible to recognize meristematic cells with loose and disassembled walls (Fig 4a), and cortical microtubules perpendicularly oriented to the wall (Fig 4b) in regions of contact with the laticifers. Laticifer projections inwards to contiguous meristematic cell, which has intermediate aspect between laticifers and meristematic cells (Fig 4c, d) are also found. The walls in the projection region are loose and exhibit signs of degradation (Fig. 4e).

In a same section of the shoot apex, the laticifer showed differences in the protoplast density and in the organelles population (Fig 3a). Some laticifers have strongly electron-dense content, numerous spherical mitochondria, plastids lacking grana and with plastoglobuli, small vacuoles with electron-dense granular content or membranous materials (cytoplasmic debris), and vacuoles fusion (Fig 3a, f). Periplasmic space containing membranes debris and electron-dense content (Fig 4f) are observed. Other laticifers have less dense content showing numerous vesicles and vacuoles with electron-dense granular material (Fig 3a, d); in the protoplast, occurs spherical mitochondria (Fig 3b), rough endoplasmic reticulum (Fig 4g), Golgi bodies (Fig 4h) and polyribosomes (Fig 4h). Vesicles derived from the dilated regions of the rough endoplasmic reticulum (Fig 4g, h) and numerous vesicles associated with the *trans*-face of Golgi bodies (Fig 4h) are observed. Vacuolated laticifers have electron-opaque inclusions surrounded by electron-dense content (latex particles) into the vacuoles (Fig 3b) or, less commonly, disperse in the cytoplasm.

In the most differentiated portions of the shoot apex occurs laticifers with large central vacuole and reduced peripheral cytoplasm (Fig 4i). The vacuole contains

membrane debris, flocculated material and latex particles (Fig 4i). The cytoplasm has the above mentioned organelles.

Immunolocalization of pectin epitopes and β -D-glucans

Embryo (Figs. 5, 6)

JIM7 (HGs with high degree of methyl-esterification) labeled uniformly and strongly whole cell walls of the tissues, including laticifers (Fig 5b, Table 1). JIM5 (HGs with low degree of methyl-esterification) present the same labeling for all tissues, but it was less intense (Fig 5c) than JIM7 labeling (Fig 5b). Immunogold electron microscopy revealed that JIM7 shows uniform distribution throughout the cell wall (Fig 5h), while JIM5 is more abundant in the middle lamella (Fig 5i).

LM5 and LM6 which recognize, respectively, galactans and arabinans side chains associated with RG-I presented distinct spatial labeling (Fig 5d, e, g). While LM5 epitope were detected in some cell walls of procambial cells after pre-treatment with pectate lyase (Fig 5g), LM6 epitope labeled the walls of different cell types, including laticifers (Fig 5d, e, Table 1). The preincubated sections with pectate lyase labeled strongly (Fig 5d). Samples that did not receive the pre-treatment exhibited no labeling for LM5 (data not shown). Immunogold analysis by TEM showed no labeling with LM5 on the laticifers or meristematic cells walls in any of the treatments (Fig 5j). The immunolocalization analysis with colloidal gold for the LM6 antibody did not show the expected labeling pattern (Fig 5k).

BS400-2 (callose) produced a punctuated labeling pattern in the walls of laticifers and the ground meristem cells adjacent and nonadjacent to laticifers (Fig 6a-c, Table 1). The pre-fixed samples with DDG (Fig 6c) showed the same labeling pattern for BS400-2 whose samples were not pre-fixed (Fig 6a, b). In TEM analysis,

BS400-2 labeled the region where plasmodesmata occur (Fig 6d-g), which would explain the punctuated pattern observed in fluorescence. The samples incubated with BS400-3 (MLG) showed no labeling (Fig 6i, j, Table 1).

Sections with omission of the primary antibodies JIM5, JIM 7, LM5, LM6, BS400-2 and BS400-3 did not show labeling by fluorochrome FITC or colloidal gold in samples analyzed under fluorescence or transmission electron microscopy, respectively (data not shown).

Shoot apex in plant (Figs. 7-10)

JIM7 and JIM5 antibodies showed different labeling patterns. JIM7 epitope labeled uniformly and strongly the walls of all the cells, including laticifers (7b, Table 1). JIM5 epitope revealed not uniform labeling pattern in the undifferentiated and differentiated portions of the shoot apex (Fig 7c). In the most apical region (undifferentiated portion), the laticifer walls showed more intense labeling than the adjacent meristematic cells (Fig 7e, Table 1). In the more differentiated portions the laticifers labeling was similar to the adjacent cells (Fig 7g, h; Table 1). In immunolocalization analysis by TEM, the JIM7 labeling is distributed throughout the cell wall of laticifers and adjacent cells (Fig 7h); while, the JIM5 labeling is more abundant in the middle lamella (Fig 7i), mainly in swollen regions where laticifers are in contact with two or more cells.

LM5 and LM6 presented distinct spatial labeling (Fig 8). LM5 weakly labeled galactans epitope in the walls of meristematic cells (Fig 8b) and labeled more intensely parenchyma cell walls (Fig 8g). In undifferentiated tissues, the laticifer walls exhibited weak or no labeling for LM5 (Fig 8b); while the walls of laticifers in differentiated tissues exhibited similar labeling pattern than adjacent parenchyma cells

(Fig 8g, Table 1). In contrast to LM5, LM6 labeled arabinans epitope more strongly in the laticifer walls than in the walls of adjacent meristematic cells (Fig 8c, Table 1). Labeling with LM6 was more intense in the meristematic cells (Fig 8c) than in the differentiated cells (Fig 8h). In undifferentiated portion of the shoot apex, the laticifer walls labeling became similar to the surrounding cells (Fig 8c, Table 1). Sections previously incubated with pectate lyase (Fig 8b, 8d) exhibit same labeling pattern to the sections that have not gone through this step and were incubated with LM5 (Fig 8e) or LM6 (Fig 8c). However more intense fluorescence was detected to LM5 in preincubated sections (Fig 8b, g).

The pattern observed using immunofluorescence was confirmed by immunogold electron microscopy to binding for LM5 (Fig 9a-e). LM5 epitopes occur within the laticifer walls in undifferentiated tissue (Fig 9a, b). However, the abundance of this epitope is lower compared to the adjacent meristematic cell (Fig 9a, b) and even lower between laticifer common walls (Fig 9c). In the region where the tissues are differentiated, the LM5 labeling for the laticifers walls is similar to the adjacent parenchyma cells (Fig 9e). It is interesting observe that LM5 labeling have association with an inner cell wall layer near to the plasma membrane (Fig 9a, b, d).

Immunolocalization with gold analyzes by TEM for LM6 did not exhibit the expected labeling (Fig 9f).

BS400-2 exhibited a punctuated labeling pattern in the most cell walls (Fig 10a-g). The walls of laticifers and adjacent cells were labeled with less intensity compared to non-adjacent cells, both in proximal (Fig 10a, b) and distal (Fig 10d, e, f) regions of the apical meristem. Prefixed samples with DDG showed the same labeling pattern for BS400-2 (Fig 10f) than the samples that were not prefixed. BS400-2 showed intense labeling in the walls of sieve tube elements (Fig 10d-f), especially in

the sieve plate. The analysis of immunolocalization by TEM did not exhibited labeling for BS400-2 in common walls of laticifers and adjacent cells (Fig 10c). Binding for this epitope was observed in regions with plasmodesmata in parenchyma (Fig 10g) or meristematic cell walls.

Sections incubated with BS400-3 showed no labeling for this epitope in fluorescence (Fig 10h, i) or immunogold (Fig 10j) analyses.

The incubation process with goat anti-mouse IgG (*whole molecule*)-Gold to analysis by TEM of BS-4002 and BS-4003, possibly, (Fig 10c, g, j) had resulted in a large amount of particulate material observed in the sections.

Sections with omission of the primary antibodies JIM5, JIM 7, LM5, LM6, BS400-2 and BS400-3 did not show labeling by fluorochrome FITC or by the secondary antibody conjugated with colloidal gold in samples analyzed in fluorescence or transmission electron microscopes respectively (data not shown).

Discussion

Laticifer ultrastructure

Our ultrastructural studies on laticifers of *T. catharinensis* in embryo and plant support the conclusions drawn from analyzes light microscopy [9] that the development of the laticifer system in this species involve protoplast fusion, the addition of cells and intrusive growth.

Ultrastructurally, the occurrence of intrusive growth in articulated laticifers in *T. catharinensis* is supported by some evidences as the irregular outline of laticifers, swollen middle lamella and thicker walls in the contact region of laticifer with two or more cells, and presence of thin projections of laticifers intruding among meristematic adjacent cells. Loosening of middle lamella among cells in the growth regions is

commonly described for intrusive growth [1,3]. Notably, these evidences were stronger in shoot apex of plant than mature embryo, and it seems be related with the differences in the cell differentiation rates between these developmental vegetative stages. Based on our observations, the cell incorporation is the predominant growth mechanism of laticifers in mature embryo, when the rates of multiplication and elongation cells are reduced.

Our data showed that the process of incorporation of new cells to the laticifer system differ in embryo and plant of *T. catharinensis*. In the embryo, this process is characterized by the presence of pores (which arising from enlargement of plasmodesmata) between common walls of laticifers and ground meristematic cells, and degradation of reserve material from the meristematic cell in the pore region, besides of the occurrence of cells with intermediated features between these two cell types, that occur in both embryo and plant. Similar findings were reported in articulated laticifers of the mature embryos of *Nerium oleander* [7] and *Cryptostegia grandiflora* [6], both Apocynaceae species. As described for *N. oleander* embryo, ground meristem cells have protein grains and lipid drops as storage material, while differentiated laticifers do not have these substances (due to solubilization of reserves at the beginning of the laticifers differentiation). The occurrence of protein grains and lipid drops in laticifers indicate the recent incorporation of a cell to the laticifer system [7].

In the plant shoot apex of *T. catharinensis*, the process of cell incorporation to laticifer system involves the loose and disassembled of the cell walls and reorientation of the cortical microtubules of the incorporated meristematic cell. In addition, the presence of cells sharing features of laticifers and meristematic cells, connected by laticifer projections inwards meristematic cells, is a strong evidence of the inducing

action of laticifers on other cells, as proposed by Milanez [7]. This mode of cell incorporation, involving the formation of laticifer projection inwards adjacent cells, is similar to that reported for the laticifer system in the mature embryo of *N. oleander* [7]. It is important to highlight that the occurrence of laticifers in different developmental stages (revealed by ultrastructural differences between laticifers) in the same shoot apex in *T. catharinensis* is an indicative of continuous addition of new cells to the laticifer system.

The occurrence of obstructed plasmodesmata in laticifers of plant of *T. catharinensis* has been associated with intrusive growth [1], because the symplastic connections are interrupted during the laticifer intrusion between adjacent cells. In the other hand, broken plasmodesmata have been also reported to articulated laticifers of *Calystegia silvatica* –Convolvulaceae [31] and *Hevea brasiliensis* –Euphorbiaceae [32], that do not have intrusive growth.

One of the roles assigned to the laticifers is the isolation of chemicals defense of sensitive tissues [33]. We have supposed that the decrease of symplastic connections between laticifers and the adjacent cells, as observed in *T. catharinensis*, may contribute to the isolation of toxic substances. The electron-lucent materials within the common walls between laticifers and adjacent meristematic cells in *T. catharinensis* may act as an aploplastic barrier that assists in the isolation of chemicals in laticifers.

The protoplast arrangement of mature laticifer in plant of *T. catharinensis*, characterized by well-developed central vacuole and peripheral cytoplasm, is usually described in both articulated and nonarticulated laticifers [1,7,31,34,35,36,37,38]. The dilation of portions of rough endoplasmatic reticulum in laticifers of *T. catharinensis* contributes to the vacuome development as reported to others latex-bearing plants

[34,35,38,39]. Simultaneously, vesicles derived from the Golgi bodies also contribute to increase of vacuolation in laticifers of *T. catharinensis*.

Immunolocalization of pectin epitopes and β -D-glucans

We evaluated the occurrence and distribution of JIM7 and JIM5, which recognize HGs with high and low degrees of methyl-esterification, respectively; LM5 and LM6, which recognize, respectively, galactans and arabinans side chains associated with RG-I; and BS400-2 and BS400-3, which recognize (1→3)- β -D-glucans, including callose, and MLG, respectively.

Our immunocytochemical studies reveal that there are difference regarding the presence/abundance of pectin epitopes and callose between the cell walls of the laticifers and adjacent cells; between laticifer walls in proximal or distal regions of the apical meristem in the shoot apex of plants; as well as between laticifer walls of the shoot apex of the plant or the mature embryo *T. catharinensis*.

HGs comprises around 65% of cell wall pectins [16] and has roles related to primary cell wall assembly and cell extension, cell wall matrix porosity and plant defense responses [40].

In the shoot apex of plant in *T. catharinensis*, low methyl-esterified HGs (recognized by JIM5) occur most abundantly in the laticifer walls than walls of the adjacent meristematic cells. Although we have not evaluated enzymatic activity in cell wall, the abundant low methyl-esterified HGs labeling may be result of the higher pectin methylesterases activity in the laticifer walls in the shoot apex of plants in *T. catharinensis*. It is interesting to highlight that the same labeling pattern for JIM5 was noticed to walls of laticifers and surrounding cells of the shoot apex of *A. speciosa*, an Apocynaceae species with nonarticulated laticifers [12,13].

Our immunogold analyses reveal that low methyl-esterification HGs occur mainly in the middle lamella, both embryo and plant of *T. catharinensis*. For Serpe et al. [12], perhaps this pectin-rich middle lamella facilitates the transport of nutrients and water across the cell walls and acts as lubricant reducing friction during growth of laticifer tips. For us, these functions of the pectin-rich middle lamella are plausible. We did not observe symplastic connections between laticifers and the adjacent cells, but we observed swollen middle lamella and JIM5 labeling regions where laticifers are in contact with two or more cells, which are possible regions of intrusive growth.

Galactans have not been detected in walls of meristematic regions [41] and they have been reported in mature cells, which must maintain some extensibility [42].

The distinct spatial labeling for LM5 ((1→4)-β-D-galactans) and LM6 ((1→5)-α-L-arabinans) in cell walls in the mature embryo of *T. catharinensis* suggest that RG-I may have different functions within the primary cell wall matrix [43]. RG-I represents 20–35% of the pectic matrix and is an alternate form of HGs, which has many side chains, mainly arabinans and galactans [16]. The no labeling of the (1→4)-β-D-galactans in cell walls in the embryo and its detection in cell walls of the plant is a indicative of the necessity of a distinct layer of the cell wall for deposition of this galactan [43], which can be absent in mature embryo cell walls in *T. catharinensis*. This distinct layer may be localized in the inner side of the cell wall [43], observed by us using LM5 immunolabeling by TEM.

The lower abundance of the (1→4)-β-D-galactans in laticifer walls localized in undifferentiated portion of the shoot apex of plant in *T. catharinensis* probably kept a less firm structure of the cell wall ensuring the flexibility required for intrusive growth. The correlation between the abundance of (1→4)-β-D-galactans with firmer tissues [43] and decrease of the porous wall architecture [44] has been noticed in other

systems. Similar findings were reported for LM5 labeling for laticifer walls in shoot apex of *A. speciosa* [12] and *Euphorbia heterophylla* (Euphorbiaceae) [14]. For Serpe et al. [14], the low levels of (1→4)-β-D-galactans in laticifers would make their walls more porous, tending to increase the extensibility of laticifer walls and facilitating the transfer of assimilates to the laticifers.

The higher abundance of the (1→5)-α-L-arabinans in laticifer walls in undifferentiated portion of the shoot apex of plant in *T. catharinensis* possibly improved flexibility of the cell wall, a favorable feature for intrusive growth. Similar findings were reported for LM6 labeling for laticifer walls in shoot apex of *A. speciosa* [12]. Pectic arabinan has been shown to be required to maintain the flexibility of guard cell walls [45,46]. The removal of the arabinans allows HG polymers to coalesce and form tightly associated structures, making the guard cells incapable of deforming in response to changes in cell turgor [45].

Sections pre-treated with pectate lyase exhibited more intense fluorescence for LM5 and LM6; and LM5 labeling of procambial cells in mature embryo (which did not observed in non pre-treated sections). Our observations showed that pectin epitopes mask epitopes recognized by LM5 and LM6, because they are cross-linking with these glycans [28,29,47].

The punctuated labeling pattern for BS400-2, which recognize callose, in the common walls of laticifers and ground meristem cells in the embryo and its no labeling in laticifers in the shoot apex in *T. catharinensis* revealed that the callose labeling coincides with the plasmodemata in both samples pre-fixed or not with DDG, excluding the presence of callose as result of the collection or fixation injuries. This finding is compatible with transmission electron microscopy analyzes that showed numerous plasmodesmata in laticifers of the embryo and their absence in laticifers of

the plant shoot apex. Additionally, the absence of callose accumulations in the adjacent cells walls to laticifers suggest that the intrusive growth does not damage the neighboring cell wall [12,14]. In *A. speciosa* [12] and *E. heterophylla* [14], common walls between laticifers adjacent cells did not labeling for BS400-2. In these species, the absence of callose in laticifer walls is associated with the nonarticulated laticifers development [14]. Callose is deposited in the forming cell plate [48] and in the nonarticulated laticifers do not occur cytokinesis [11]. Furthermore, callose is also found associated with plasmodesmata [21] and, usually, this laticifer type have no these structures [12,14].

In shoot apex of *T. catharinensis*, *A. speciosa* [12] and *E. heterophylla* [14], the level of (1→4)-β-D-galactans is much lower in laticifer walls than in other cell walls, and callose do not occur in common walls between laticifer and adjacent cells. These comparisons indicate that the intrusive growth mechanism, in articulated and nonarticulated laticifers, is resulting from similar modifications in wall features. On the other hand, the higher labeling of the low methyl-esterified HGs and (1→5)-α-L-arabinans in laticifer walls from the undifferentiated portion of the shoot apex in *T. catharinensis* are shared with *A. speciosa* [12], both Apocynaceae species. These evidences suggested that modifications in laticifer wall features, associated with intrusive growth, may also taxa inherent.

MLG, recognized by BS400-3, no labeling any cell type in *T. catharinensis*. In fact, this wall component is rare in the plant kingdom and it has been found in *Equisetum*-species and Poales [49,50].

Actively secreting laticifers occur from the mature embryo in *T. catharinensis* [9]. However, our data suggest the laticifer walls in the mature embryo are in an

“immature state”, once the wall laticifer features change during the plant development.

Conclusions

The conventional and immunocytochemical methods in light and transmission electron microscopies studies were complementary and allow us explore cell incorporation and the intrusive growth processes during the articulated laticifers development. The monoclonal antibodies used here revealed the dynamics of the cell wall during laticifer development and allow us verify that the glycans distribution in articulated laticifer walls support the same intrusive process as in nonarticulated laticifers. The ultrastructural study revealed that processes of cell incorporation to laticifer system differ in mature embryo and plant. However, in both the cases, the protoplast of the incorporated cells acquires similar features of laticifers, and the incorporation is restricted to those cells in contact with laticifers. A limitation found in this study was that we were not able to detect changes in glycan epitopes of the cell walls during its incorporation to laticifer system.

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Table, Figures and Figure Captions

Table 1. Binding of the antibodies to laticifer walls in mature embryo and plant shoot apex, comparing with adjacent cell walls, in *Tabernaemontana catharinensis* A.DC. The number of signals indicates the intensity of the reaction (+) weak, (++) moderate and (+++) intense; or (–) no labeling. LM= Leeds Monoclonal, JIM= John Innes Monoclonal, BS= Biosupplies.

Monoclonal Antibodies	Epitopes	Mature embryo – Cell walls		Shoot apex of plants – Cell walls			
		Laticifers	Ground meristem cells	Laticifers in undifferentiated portion	Meristematic cells	Laticifers in differentiated portion	Parenchyma cells
JIM7	HGs with high degree of methyl-esterification	++	++	+++	+++	+++	+++
JIM5	HGs with low degree of methyl-esterification	+	+	+++	+	++	++
LM5	(1→4)-β-D-galactans	–	–	–	+	++	++
LM6	(1→5)-α-L-arabinans	+	+	+++	+	+	+
BS400-2	Callose	+	+	–	–	–	+
BS400-3	MLG – (1→3, 1→4)-β-D-glucans	–	–	–	–	–	–

Fig 1. Ultrastructural characterization of laticifers in the mature embryo in *T. catharinensis*.

(a) Laticifer (*la*) has reduced cytoplasm interspersed with abundant lipid and protein bodies. (b) Laticifer with irregular in outline and projections where there is contact with two or more cells. (c) Laticifer walls with loose structure and striate texture. (d) Laticifer has plasmodesmata and signs of degradation of the reserve material. (e-f) Enlarged plasmodesmata channels (e) and pores (f) connecting the laticifer to an adjacent meristematic cell. *la*= laticifer; *gm*= ground meristem cell.

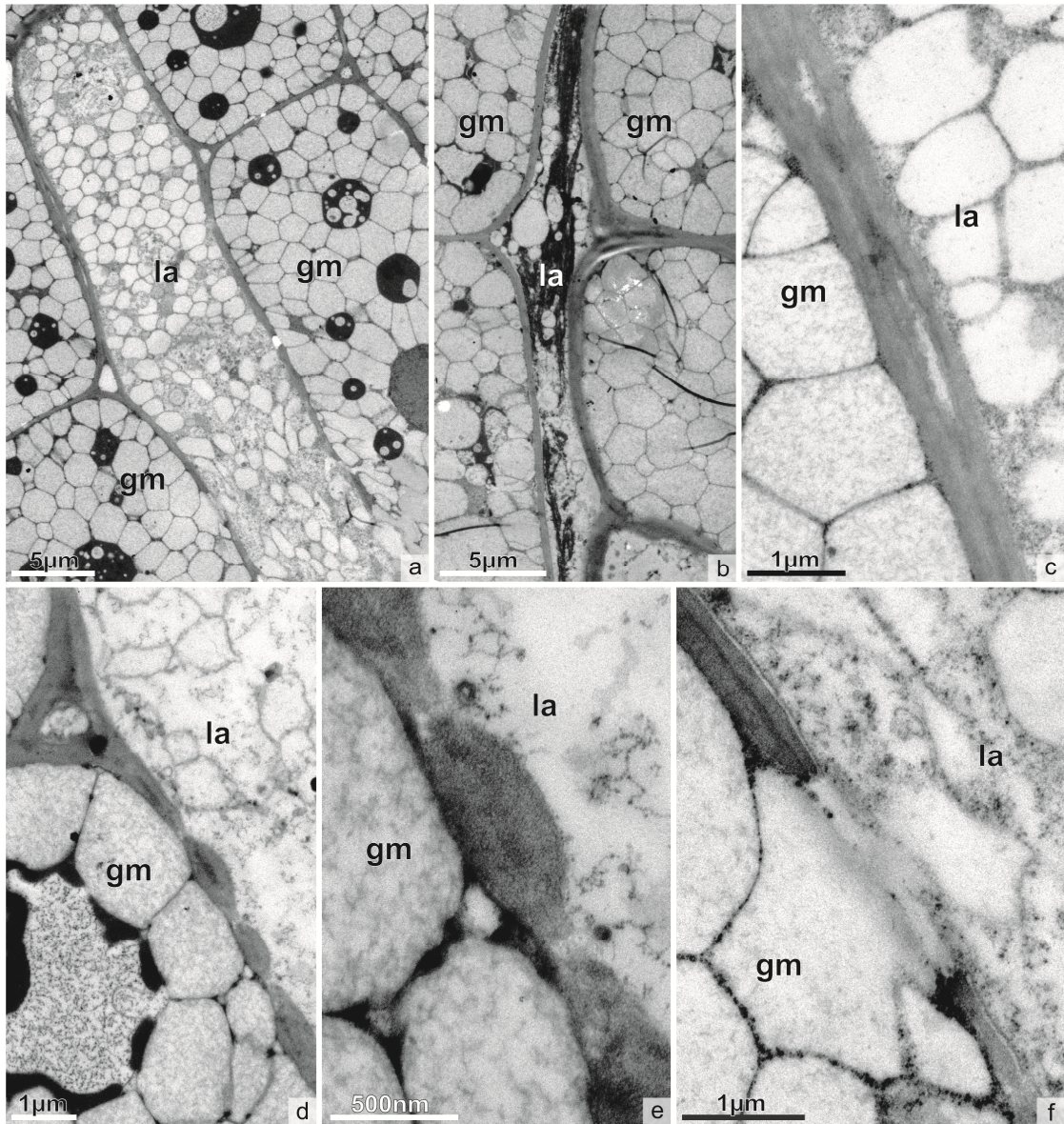


Fig 2. Ultrastructural characterization of laticifers in the mature embryo in *T. catharinensis*.

(a) Ground meristem cell (*gm*), which is in contact with a laticifer, have signs of degradation of the reserve material (*asterisk*) near the pore region. (b-c) Laticifer protoplast containing electron-dense strands of endoplasmic reticulum (*er*), small vacuoles (*va*), and mitochondria (*mi*). (d) Elongated cell irregular in outline and tip intrusive growth. Note Golgi body with adjacent vesicles (*inset*). *la*= laticifer; *gm*= ground meristem cell. *la*= laticifer; *gm*= ground meristem cell.

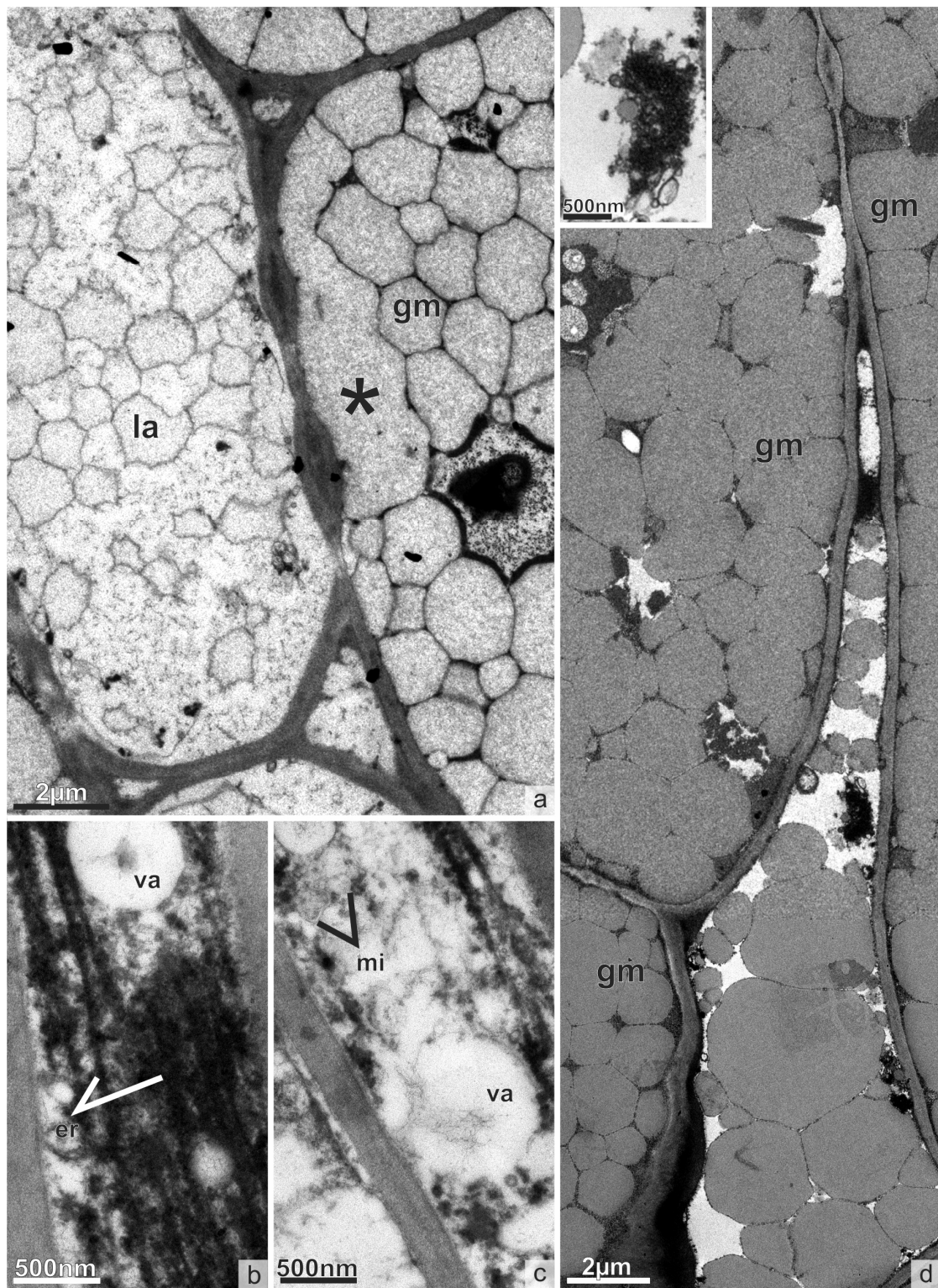


Fig 3. Ultrastructural characterization of laticifers in the shoot apex of plant in *T. catharinensis*.

(a) Laticifers with distinct ultrastructural features in a same shoot apex. (b) Laticifer irregular in outline with projections where there is contact with two or more cells. Note electron-opaque inclusions surrounded by electron-dense content ($\diamond$) disperse in the cytoplasm and mitochondria (*mi*). (c) Thick Laticifer wall and swollen middle lamella in regions where they are in contact with two or more cells. Note plastid in parenchyma cell containing electron-lucent materials (*inset*). (d) Electron-lucent materials (*arrowheads*) and unusual electron-dense fibrillar arrangements (*arrow*) within the common walls between laticifer and adjacent cell. Periplasmic space containing membranes debris and electron-dense material. Laticifer protoplast filled with numerous small vacuoles containing electron-dense granular material. (e) Peculiar electron-dense swellings (*arrow*) within the wall of the laticifer adjacent cell. (f) Descontinuous cell walls between laticifers. Laticifer cytoplasm with mitochondria (*mi*), plastids (*pl*) lacking grana and with plastoglobuli, small vacuoles (*va*) with electron-dense granular content or membranous materials. (g-h) Elongated and thin projection of laticifer tips (*markers*) among the adjacent cells. (i) Detail of region within the broken rectangle in (h) showing cortical microtubules perpendicularly oriented in the meristematic cells in contact with the laticifer projection. *la*= laticifer; *mc*= meristematic cell; *pa*= parenchyma cell.

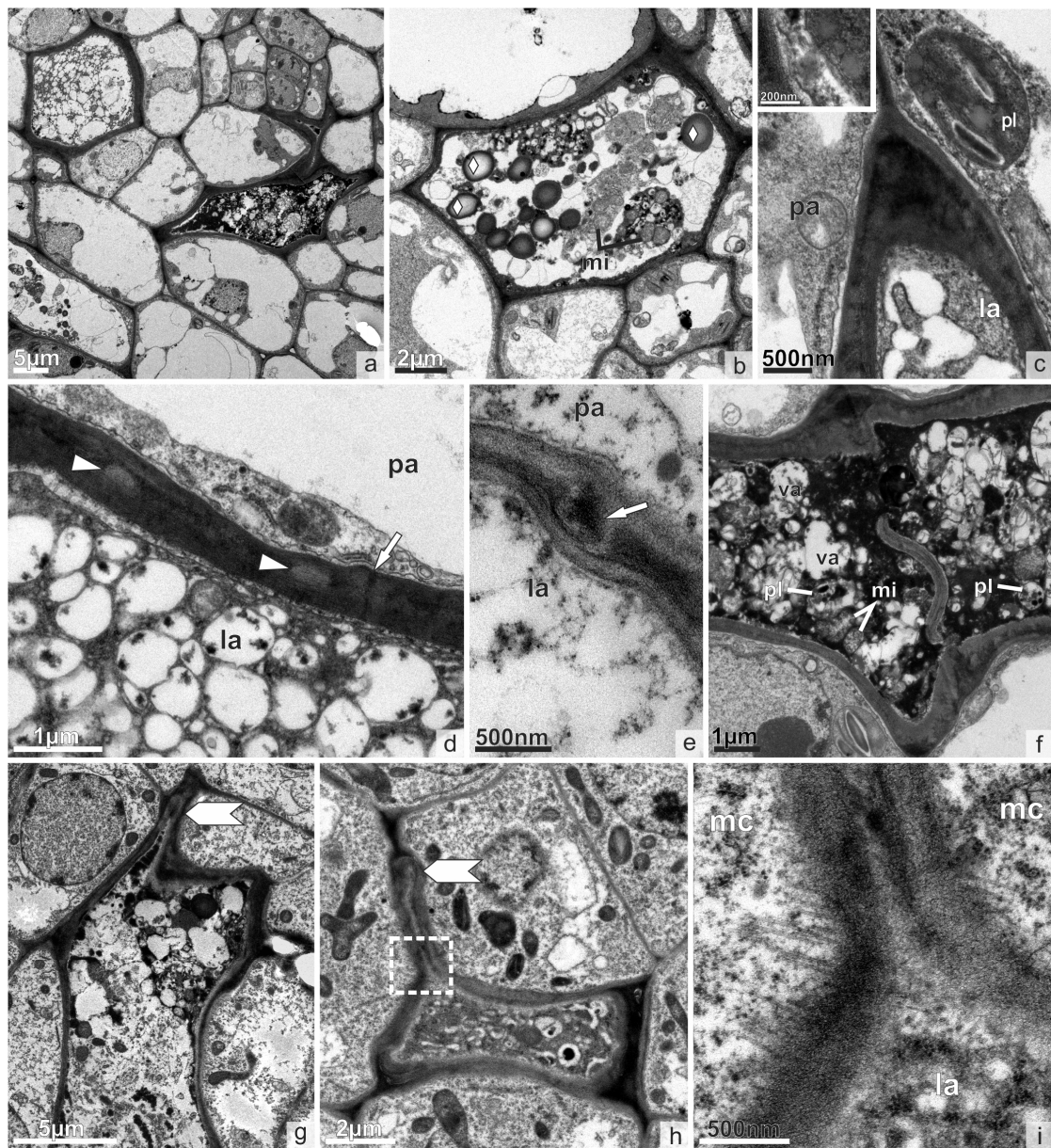


Fig 4. Ultrastructural characterization of laticifers in the shoot apex of plant in *T. catharinensis*.

(a-e) Meristematic cells incorporated to the laticifer system. (a-b) Meristematic cell with loose and disassembled walls (a) and cortical microtubules perpendicularly oriented to the wall in regions of contact with the laticifer (c-e). Laticifer projections inwards to contiguous meristematic cells. Note that intermediate aspect between laticifers and meristematic cells in the incorporated cell. (e) Loose and disassembled cell wall in the region of the laticifer projection. (f) Periplasmic space containing membranes debris and electron-dense material in laticifer. (g) Vesicles derived from the dilated regions of the rough endoplasmic reticulum (*asterisk*). (h) Golgi body (*gb*) with vesicles associated. Note dilated regions of the rough endoplasmic reticulum (*asterisk*) near the vesicles from Golgi body and polyribosomes dispersed in the cytoplasm. (i) Section non stained uranyl acetate and lead citrate. Portion differentiated of the shoot apex. Laticifer with a large central vacuole and reduced peripheral cytoplasm. Note membrane debris, flocculated material and latex particles within the vacuole. *la*= laticifer; *pa*= parenchyma cell.

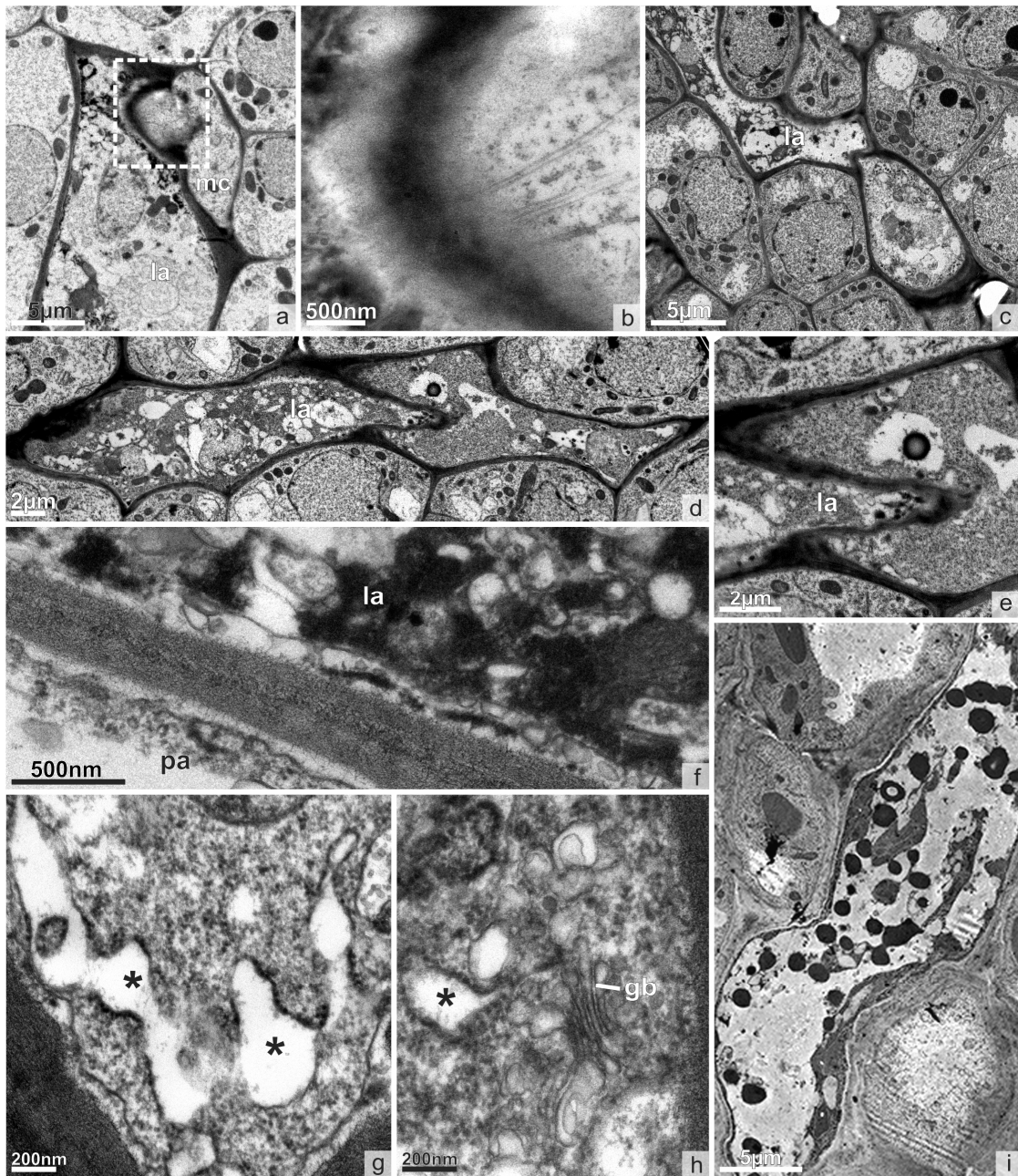


Fig 5. Immunolocalization of pectin epitopes in the walls of laticifers and adjacent cells in the mature embryo in *T. catharinensis*.

(a-g) Longitudinal sections through the cotyledonary node. (a) Light microscopy. Toluidone Blue stained section. (b-g) Immunofluorescence microscopy. (b) JIM7 labeled whole cell walls of the tissues, including laticifers. (c) JIM5 labeled weakly the tissues, including laticifers. (d-e) LM6 labeled in the walls of laticifers and adjacent cells. Section preincubated with pectate lyase labeled more strongly (d) than without this step (e). (f) Calcofluor white stained section showing the full extent of cell walls. (g) LM5 labeled after incubation with pectate lyase. Note no labeled laticifer walls (*arrow*) and procambium cell wall labeled. (h-k) Immunogold labeling for MET. (h) JIM7 labeled uniformly distributed throughout the cell wall. (i) JIM5 labeled in the middle lamella. (j-k) No labeled for LM5 (j) and LM6 (k). *la* or *arrow*= laticifer, *gm*= ground meristem cell.

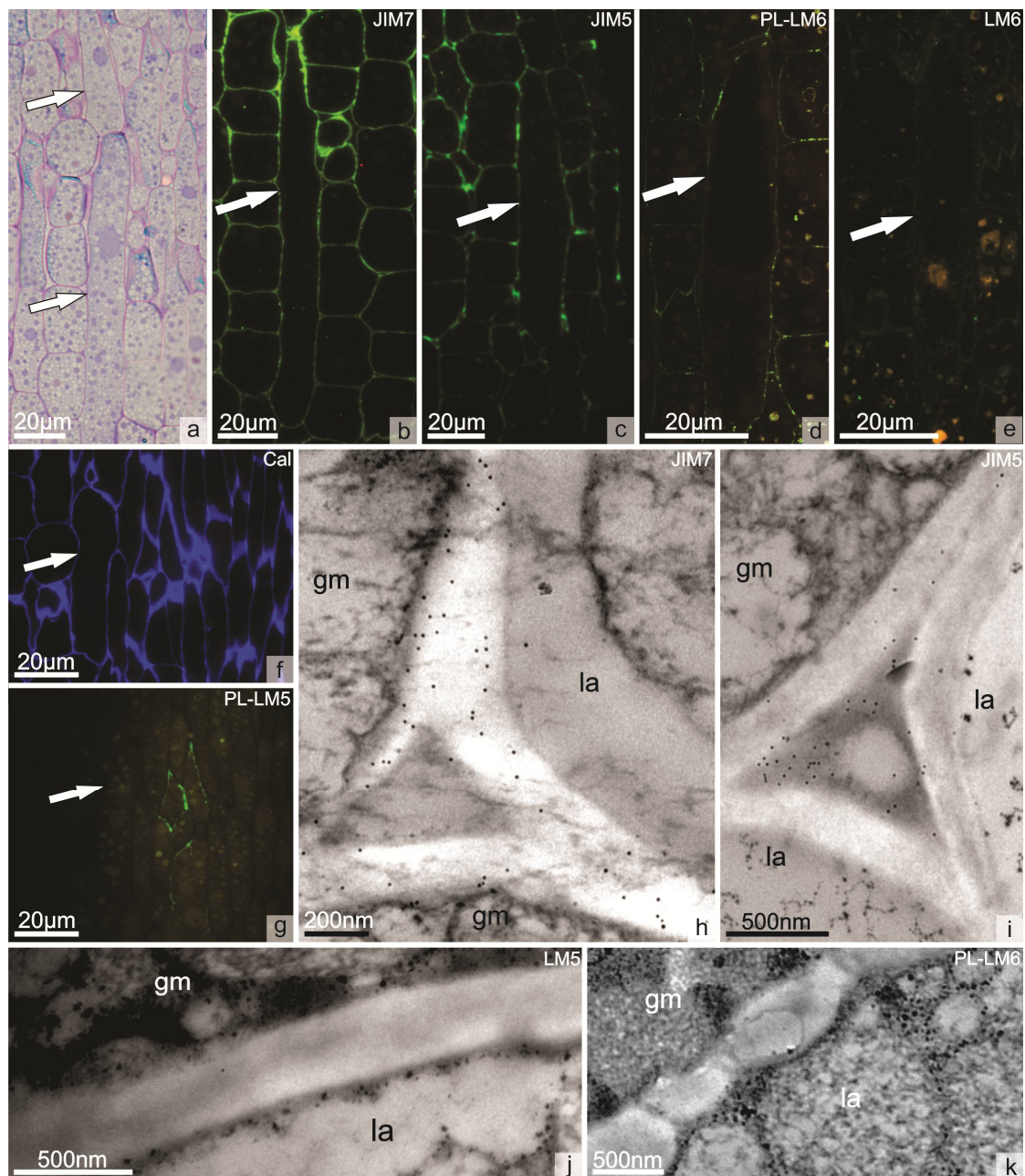


Fig 6. Immunolocalization of β -D-glucans in the walls of laticifers and adjacent cells in the mature embryo in *T. catharinensis*.

(a-c) Immunofluorescence microscopy. BS400-2 labeled. Note punctuated labeling pattern in the walls of laticifers, adjacent and nonadjacent meristematic cells. The prefixation with DDG (c) did not change the pattern of labeling. (d-g) Immunogold labeling for MET. BS400-2 labeling plasmodesmata region. (h-i) Immunofluorescence microscopy. Calcofluor white stained section (h) and no labeled for BS400-3(i). Immunogold labeling for MET. No BS400-3 labeling. *la* or *arrow*= laticifer, *gm*= ground meristem cell.

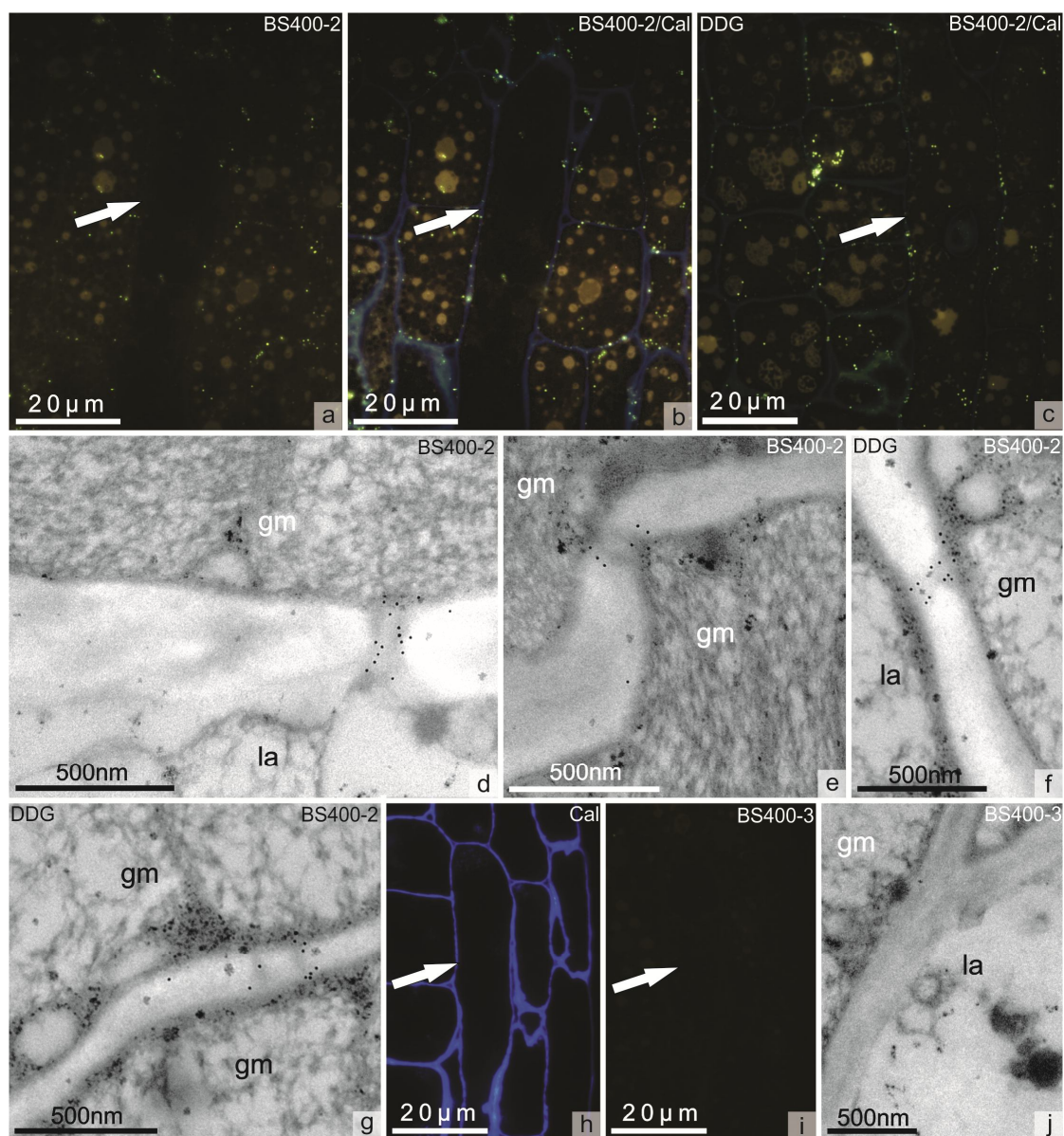


Fig 7. Immunolocalization of homogalacturonans with high (JIM7) and low (JIM5) degrees of methyl-esterification in the walls of laticifers and adjacent cells in the shoot apex of plant in *T. catharinensis*.

(a-g) Longitudinal sections. (a, d, f) Light microscopy. Toluidone Blue stained sections. (a, b, e, g) Immunofluorescence microscopy. (b) JIM7 labeled intensely different tissues, including laticifers (*arrows*). (c) JIM5 labeled weakly undifferentiated portion (*circle*) and moderately differentiated portions. (e) Laticifer in undifferentiated portion labeling for JIM5 more intensely than meristematic cells, mainly in region where it is in contact with two or more cells. (g) Laticifer in differentiated portion labeling similarly than other cells for JIM5. (h-i) Immunogold labeling for MET. (h) JIM7 uniformly distributed throughout the cell wall. (i) JIM5 labeled in the swollen middle lamella. *la* or *arrow*= laticifer, *mc*= meristematic cell.

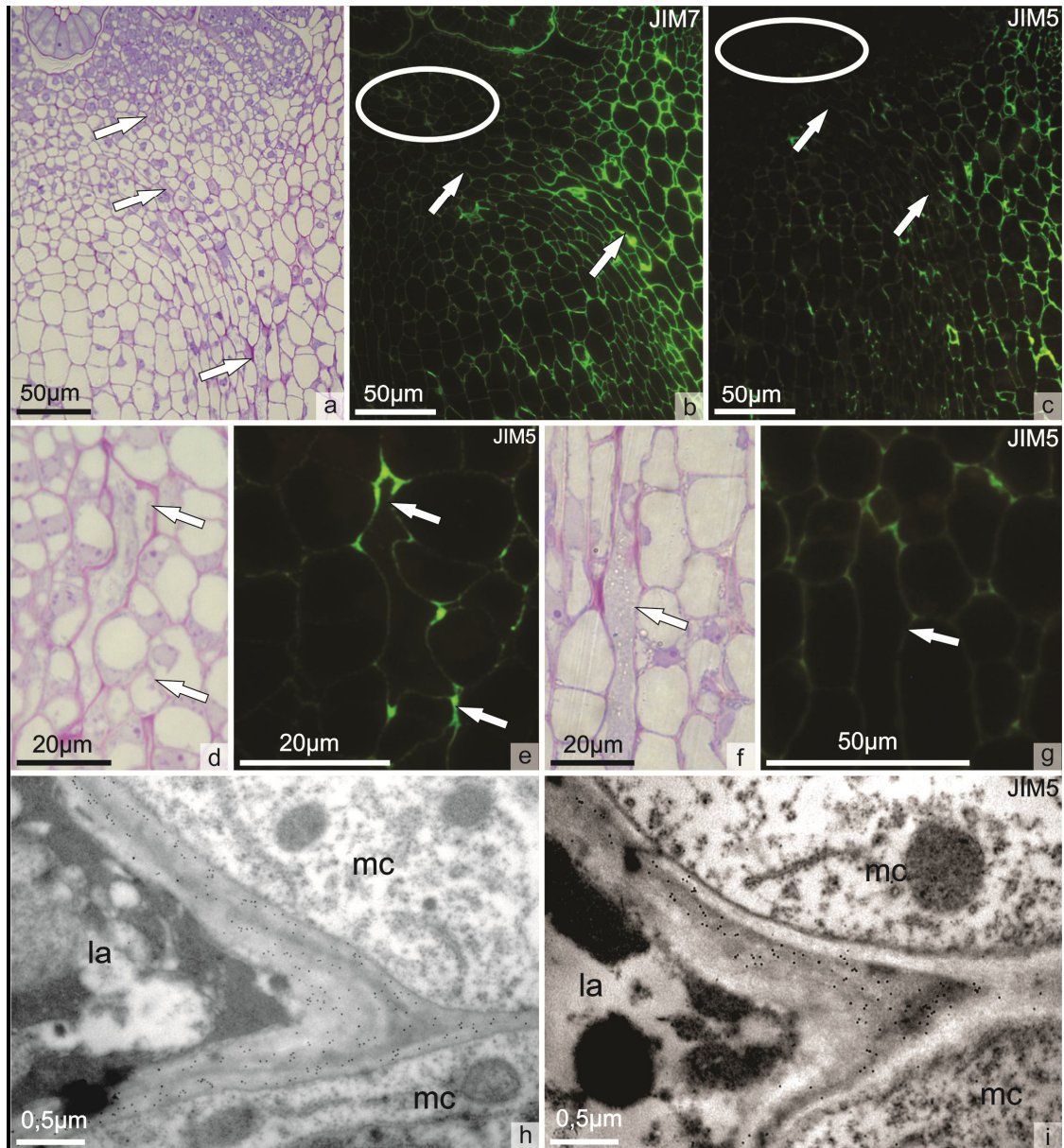


Fig 8. Immunofluorescence of galactans (LM5) and arabinans (LM6) side chains associated with rhamnogactunan I in the walls of laticifers and adjacent cells in the shoot apex of plant in *T. catharinensis*.

(a-h) Longitudinal sections. (a, f) Light microscopy. Toluidone Blue stained sections. (a-e) Laticifers in undifferentiated portions of the shoot apex. (b) LM5 labeling after incubation with pectate lyase. Laticifer walls (*headarrows*) laticifer walls no labeled. (c) LM6 labeled more intensely walls of laticifers than meristematic cells. (d) LM6 labeled with preincubation with pectate lyase. (e) LM5 without preincubation with pectate lyase exhibit weak labeled. (f-h) Laticifers in differentiated portions of the shoot apex. (g) Similar labeling for LM5 (preincubated with pectate lyase) in the walls of laticifers and parenchyma cells. (h) Similar and weak labeling for LM6 in the walls of laticifers and parenchyma cells. *la* or *arrow*= laticifer.

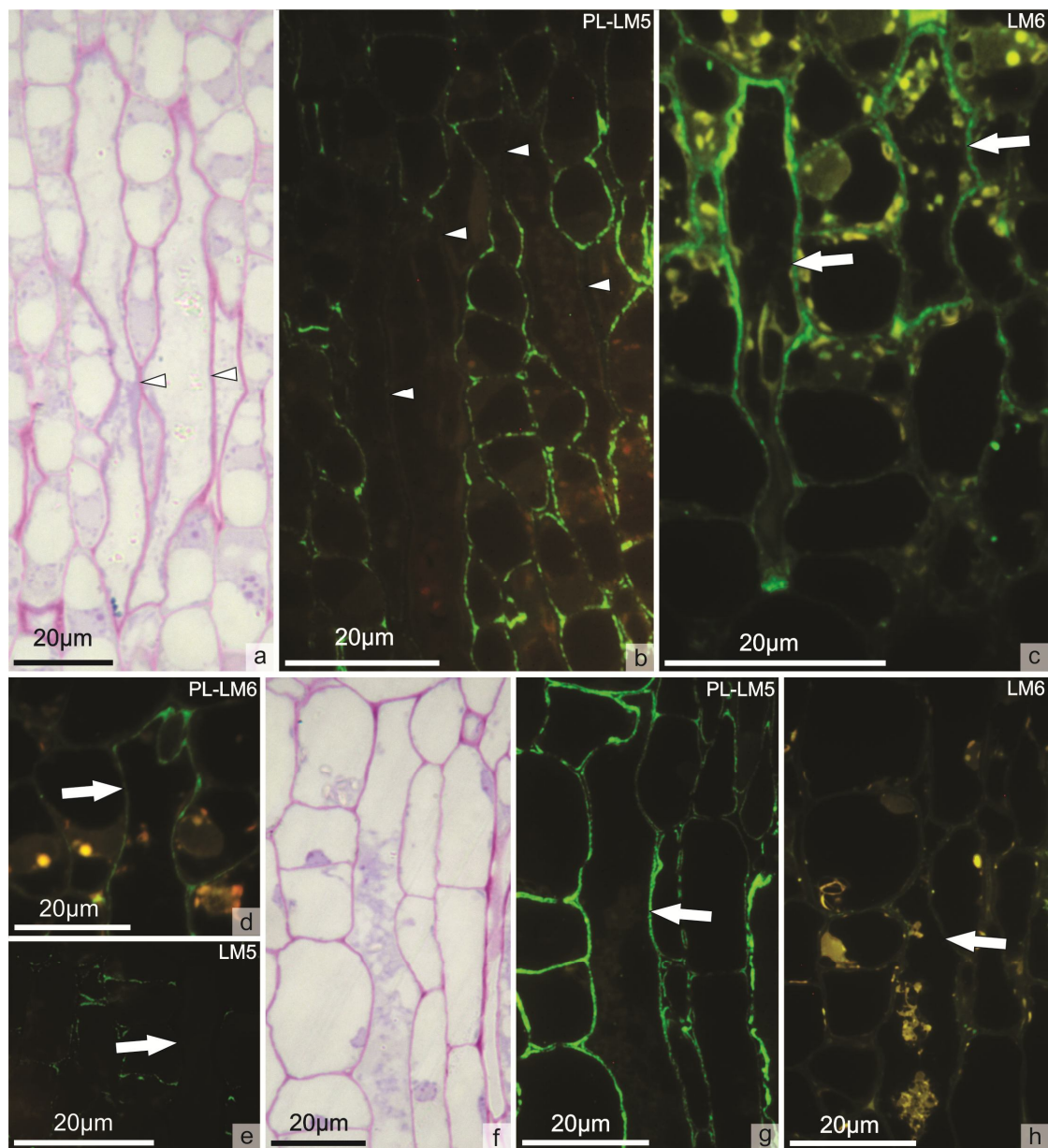


Fig 9. Immunogold labeling for MET of galactans (LM5) and arabinans (LM6) side chains associated with rhamnogactunan I in the walls of laticifers and adjacent cells in the shoot apex of plant in *T. catharinensis*.

(a-d) Undifferentiated portion of the shoot apex. (a, b) LM5 labeled inner cell wall layer near to the plasma membrane of the meristematic cells in contact with laticifers. (c) No labeling for LM5 in laticifer walls in contact. (d) LM5 labeled inner cell wall layer near to the plasma membrane of the meristematic cells in contact. (e) Differentiated portion of the shoot apex. Similar labeling for LM5 in walls of the laticifers and parenchyma cells. (f) LM6 labeled. *la*= laticifer; *mc*= meristematic cell; *pa*= parenchyma cell.

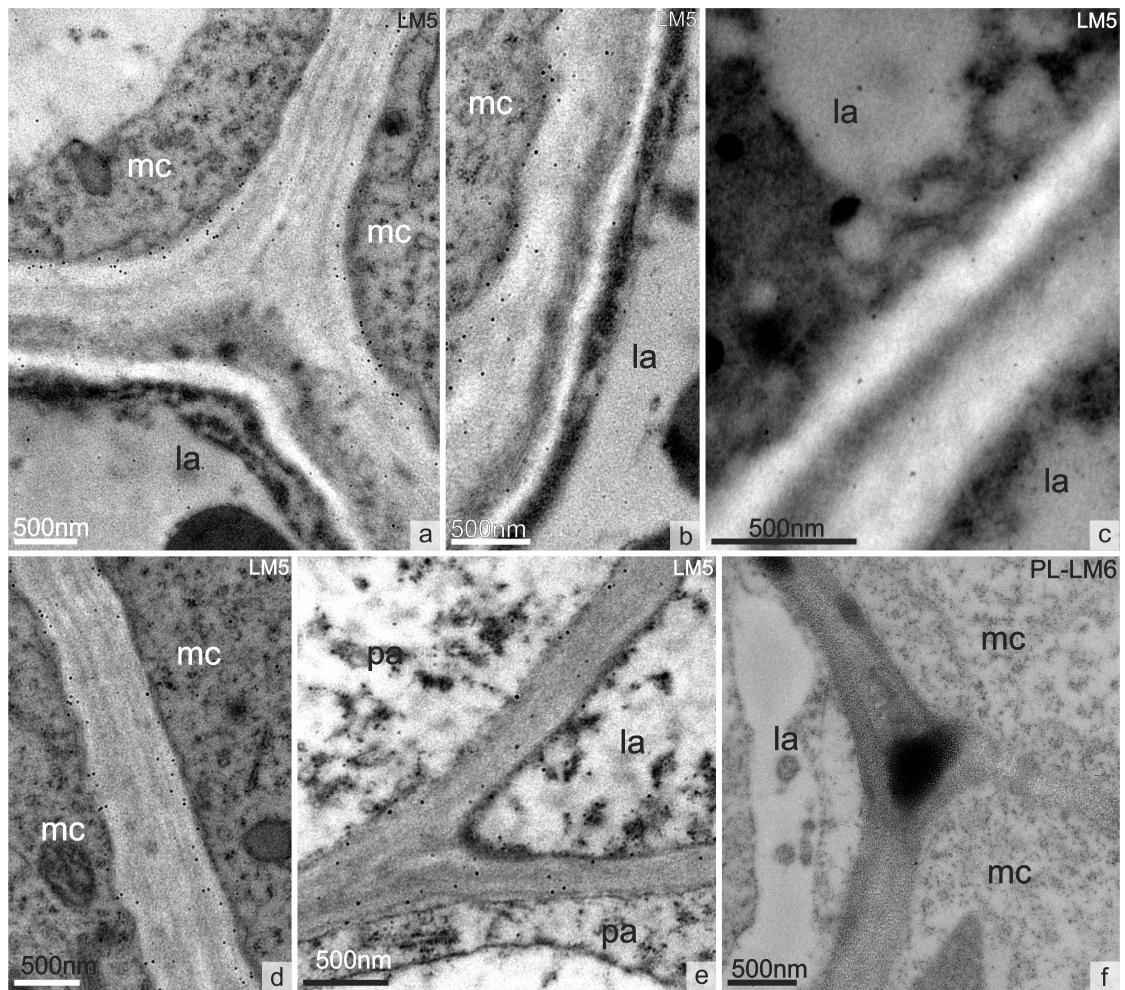
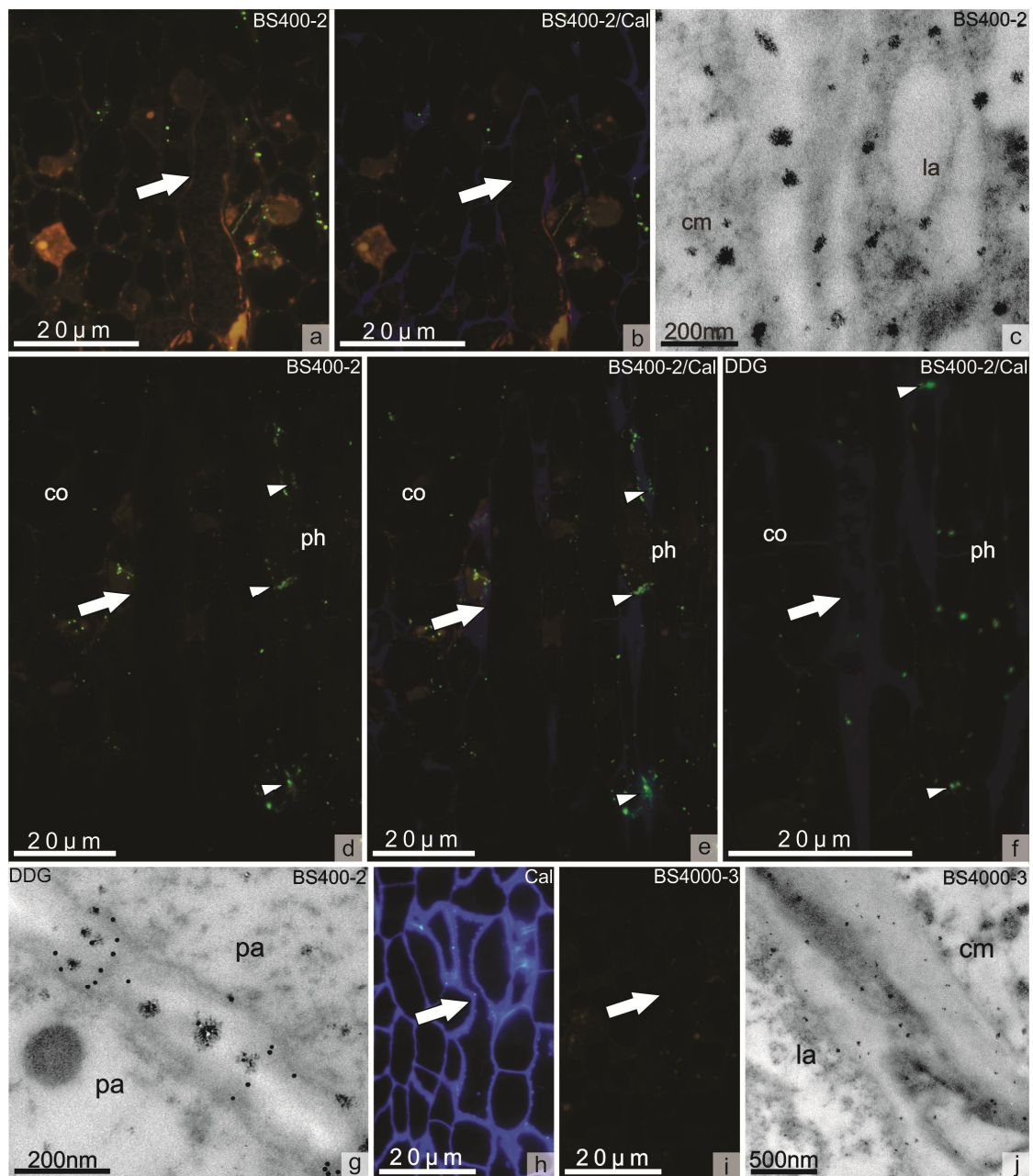


Fig 10. Immunolocalization of β -D-glucans in the walls of laticifers and adjacent cells in the shoot apex of plant in *T. catharinensis*.

(a, b, d, e, f, h, i) Immunofluorescence microscopy. **(a-c, h-i)** Undifferentiated portion of the shoot apex. **(d-g)** Differentiated portion of the shoot apex. **(a, b, d-f)** BS400-2 labeled. Note no labeling in laticifer walls and punctuated labeling pattern in the meristematic or parenchyma cell walls. **(c-d)** Immunogold labeling for MET. No labeling in the laticifers for BS400-2 **(c)** and labeling in plasmodesmata regions in parenchyma cell walls **(g)**. **(f, g)** Sections pre-fixed with DDG. **(h-j)** BS400-3 labeled. Calcofluor white stained section **(h)** and no labeled for BS400-3**(i)**. Immunogold labeling **(j)**. *la* or *arrow*= laticifer; *cm*= meristem cell; *pa*=parenchyma cell; *co*= cortex region; *ph*= phloem; *arrowhead*= sieve plate.



Influence of exogenous ethylene and jasmonic acid on the laticifers differentiation: evidence obtained from *Tabernaemontana catharinensis* (Apocynaceae) plants

Abstract

Ethylene and jasmonic acid are plant hormones released during the herbivory and may affect the differentiation of secretory structures related with plant defense, as laticifers and ducts. We investigated the influence of ethylene and acid jasmonic on the laticifer differentiation in *Tabernaemontana catharinensis* A.CD. (Apocynaceae). We sprayed solutions of the 2% ethephon, 0.1% jasmonic acid and deionized water in plants ($n = 33$) growing under controlled conditions. Forty days after the application, we collected the median portion of the internode subjacent to the shoot apex and the basal region of the stem axis, and processed according to conventional light microscopy techniques. In transverse sections, we measured of the number of laticifers and the proportion of laticifer area in relation to tissue area. The differentiation of laticifers from ground meristem (in cortex and pith) is reduced by exogenous ethylene, while jasmonic acid appears not to influence on the laticifer differentiation. Laticifer differentiation from procambium and vascular cambium seems be not influenced by ethylene or jasmonic acid. Considering the protective latex functions, we suggest that the negative effect of exogenous ethylene on laticifer differentiation in *T. catharinensis* would make the plants most vulnerable to attack by herbivores and pathogens.

Keywords: Apocynaceae, laticifer differentiation, plant regulators, ethephon, jasmonates

Introduction

Laticifers are specialized cells containing latex and forming systems that permeate various tissues of the plant body (Evert 2006). Latex is produced by 20,000 species from over 40 angiosperm families and it is also found in conifers and ferns (Lewinsohn 1991). Among tropical plant species 14% create latex, as opposed to 6% of temperate species (Lewinsohn 1991). Latex-bearing lineages have higher diversities than their sister groups and this increased in diversification has been associated with protective latex functions (Farrell et al. 1991, Agrawal and Konno 2009). Excretions of waste metabolites, coverage of damaged tissue and defense against herbivores and pathogens have been assigned as the latex's role (Konno 2011).

Several lines of evidence suggest that latex production in plants is responsive to environmental conditions, including herbivory (Agrawal and Konno 2009). The response to the plant damage can be qualitative (induction of defense-related chemical and proteins in plant latex) or quantitative (variation in the amount of latex exuded) (Konno 2001).

The production of some plant hormones is tightly regulated by internal signals during development and in response to biotic (e.g., pathogen attack) and abiotic (e.g., wounding) environmental stimuli (Taiz and Zeiger 2006). In this way, ethylene is a hormone released due to the mechanical damage and jasmonic acid, in response to chemical substances produced by the herbivore (Taiz and Zeiger 2006).

Exogenous ethylene induces the formation of the traumatic resin ducts, which are secretory structures also related to defense against herbivores and pathogens, in different species (Yamamoto and Kozłowski 1987, Fahn 1988, Hudgins and Franceschi 2004). More recently, the effect on the increase of the traumatic resin ducts has been reported by exogenous methyl jasmonate applications (Hudgins and Franceschi 2004).

Despite the ecological and economical relevance of laticifers and the existence of a considerable number of latex-producing plants, few studies have addressed on exploring the influence of plant hormones on the differentiation of laticifers. In addition, the few studies are restricted to some commercial species, especially *Hevea brasiliensis* (Euphorbiaceae), known as the Brazilian rubber tree (Hao and Hu 1984 2000). In commercial cultivation of this species, ethylene causes stimulation of latex production (Hao and Wu 1984), but does not change the number of laticifers (Hao and Hu 2000), while, jasmonic acid and its precursor, linolenic acid, increased the number of laticifers in the cortical parenchyma and secondary phloem (Hao and Hu 2000).

The influence of the plant growth regulators on the laticifers is an exciting approach that needs to be expanded to different latex-bearing plants so clarify important questions about developmental and ecological relations. Additionally, this approach can contribute to improvement programs of latex production and collect. Therefore, we investigated the differentiation of laticifers and their relation with plant growth regulators in a member of Apocynaceae. This family present pantropical distribution and contains latex-producing plants with ecological e medicinal importance (Simpson 2006) as the case of the *Tabernaemontana catharinensis* A.DC., which has been studied due to their several alkaloids with pharmacological activity (Pereira et al. 1999, Batina et al. 2000, Soares et al. 2007). In a preliminary study on *T. catharinensis*, comparing different organs and stages of vegetative growth, we found articulated anastomosing laticifers with intrusive growth originate from the ground meristem and procambium (primary plant body) and cambial fusiform cells (secondary plant body) (Canaveze and Machado *in press*).

Here, we conducted an anatomical study of the primary and secondary bodies of *T. catharinensis* plants maintained under controlled conditions and investigated the influence of ethylene and acid jasmonic on the laticifers differentiation. We focus on (1)

number of laticifers within the different tissues and (2) proportion of the area of the laticifers in relation to the tissue area.

Materials and methods

Plant Growth

To obtain plants, we collected mature fruits from *T. catharinensis* plants growing in areas of cerrado (savannah-like vegetation) in the municipalities of Botucatu (22°42'21.4''S and 48°18'23.7''W) and Pratânia (22°48'48.8''S and 48°44'33.7''W), in the state of São Paulo, Brazil, from April in 2013. In the study areas, *T. catharinensis* is a shrub or treelet with 1-8m in length.

After manual removal of the aril, we placed the seeds ($n = 600$) in acrylic boxes lined with wet filter paper at $25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ under fluorescent lamps ($460\text{ }\mu\text{mol.m}^{-2}.\text{s}^{-1}$ photon flux density; 12:12 photoperiod) in germination chambers. The seedlings were transferred to plastic tubes containing vermiculite and were kept under the same controlled light and temperature regimens until expansion of the one or two metaphyll pair (metaphylls are definitive mature leaves – Duke 1965). Then, we transferred these plants ($n = 100$) to plastic bags containing 5L of substrate (1:1 v/v – soil : C 0.7-II Carolina substrate) and kept these plants in green house with controlled temperature and humidity (around of $800\mu\text{mol.m}^{-2}.\text{s}^{-1}$ photon flux density; 25°C; 70% relative humidity). When necessary, the seedlings or plants were watered with a nutritive solution (Hoagland and Arnon 1950).

Plant growth regulators treatments

To application of the growth regulators, we selected homogeneous and healthy plants with 15-month-old ($n= 33$). In this stage, plants have 50cm in length. The shoot is a

sympodial axis with dichasial branching, and opposite-decussate phyllotaxy. The proximal portion of the axis (around 1cm in diameter) have secondary thickening.

We used the higher concentrations tested by Hao and Wu (2000) to jasmonic acid (J2500 Sigma) and ethephon, an ethylene-releasing agent, (2-chloroethylphosphonic acid, Ethrel-240 Rhodia Agro, Rhône-Poulenc), which were 0.1% and 2%, respectively, and deionized water. 0.25% vegetable oil (Natur'l oil, Stoller of Brazil) was used in the three treatments as adhesive spreader. We applied the treatments towards to the leaves with the aid of CO₂ pressurized backpack sprayer operating at constant pressure. We chose this type of application because of the small stem diameter (~1cm) and by its satisfactory results (Rasmann et al. 2009).

We marked with cotton cord two shoot apices per plant and accompanied their development. Forty days after the application of growth regulators the marked internodes and their corresponding leaves were expanded, and we collected the median portion of the marked internode, subjacent to the shoot apex, and the basal region of the stem axis at 10cm of the substrate.

Anatomical analysis

We fixed the samples (5mm long) in FAA 50 (Johansen 1940) for 48 h at 25 °C. The materials were dehydrated in an ethanol series and embedded in methacrylate resin (Leica Microsystems, Heidelberg, Germany). Transverse and longitudinal sections (5 µm thick) were made using a rotatory microtome, double stained with Sudan IV (Johansen 1940) and 0.05% Toluidine blue, pH 4.7 (O'Brien et al. 1964). The observations and image documentation were made using a microscope (Leica DMR) coupled with a digital camera (Leica DFC 425)

Measurements

The measurements were made on transverse sections using digital imaging software (LAS - Leica).

In the internode subjacent the shoot apex, we measured: 1) number of laticifers within 0.2mm^2 of the cortical parenchyma, 2) proportion of the area of the laticifers in relation to the cortical parenchyma area, 3) number of laticifers within 0.1mm^2 of the pericycle and primary phloem, 4) proportion of the area of the laticifers in relation to the area of the pericycle and primary phloem, 5) number of laticifers within 0.2mm^2 intraxylary phloem (phloem internal to the xylem) and medullary parenchyma and 6) proportion of the area of the laticifers in relation to the area of the intraxylary phloem and medullary parenchyma. In the basal region of the stem, we made de measurements in the inner region of the bark, which correspond to the secondary phloem, and we measured 7) number of laticifers within 0.3mm^2 of the secondary phloem and 8) proportion of the area of the laticifers in relation to the area of the secondary phloem.

We compared all treatments (0.1% jasmonic acid, 2% ethephon and deionized water) with eleven repetitions for each above mentioned measurement using analyses of variance (ANOVA) followed by Tukey's test at significance level $\alpha=0,05$. When necessary, we transformed the data by square root (number of laticifers) or $\log_{10}$ (proportion of area) to supply the test assumptions.

Results

Effects of regulators on plant development

Two days after application of regulators, the plants treated with 2% ethephon showed signs of phytotoxicity. The leaves close to the shoot apex wilted and had necrotic

spots (Fig 1a). In the following days occurred yellowing and fall of the leaves (Figure 1b, c) and formation of cracks in the stem bark (Fig 1d). Fifth days after ethephon application, plants lost all leaves. Resprouting close the basal portion of the stem (Fig 1e) was observed fifteen days after application. The resprouts exhibited usual development.

Plants treated with 0.1% jasmonic acid and deionized water exhibited no signals of phytotoxicity and they had a usual development (Fig 1f).

Laticifer distribution and structural aspects

In the primary plant body, laticifers occur immersed in parenchyma tissues (cortical and medullary) (Fig 2a-e) and in the primary phloem (Fig 2a, f), including intraxylary phloem (Fig 2g). In the secondary plant body, laticifers occur associated with secondary phloem (Fig 3a-c).

In the primary body, laticifers with variable diameter were observed side by side (Fig 2b). Adjacent axial chains of laticifers occur commonly (Fig 2c, d) and signals of fusion between adjacent chains were observed in both longitudinal (Fig 2e) and transverse sections (Fig 2f, g). In transverse sections, laticifers were distinguished from other cells mainly by their protoplast features, orange stained or blue stained with the use of Sudan IV (Fig 2c) and Toluidine blue (Fig 2b), respectively.

The bark, observed in transverse section through the basal region of the axis, comprises the periderm, cortex, pericyclic fibers, primary phloem and secondary phloem (Fig 3a, b). In the secondary phloem, laticifers were distinguished from other cells in transverse section by their angular outline and absence or fewer starch grains compared with the axial and radial parenchyma cells (Fig 3b).

Plant growth regulators on laticifer differentiation

The number of laticifers and the proportion of laticifer area in relation to the tissues varied among the different treatments (Table 1, Fig 4). In plants treated with ethephon, the number of laticifers in the cortical parenchyma is significantly smaller than those treated with jasmonic acid ($p<0.05$) (Fig 4a). In the intraxylary phloem and medullary parenchyma, the number of laticifers in plants treated with ethephon is significantly smaller than those treated with deionized water ($p<0.05$) (Fig 4c).

The proportion of laticifer area in relation to cortex was not change by the different treatments ($p>0.05$) (Fig 4a). With the ethephon application there was a decreasing in the proportion of laticifer area in relation to intraxylary phloem and medullary parenchyma area in regarding to jasmonic acid and deionized water ($p< 0.001$) (Fig 4c).

The number of laticifers and the proportion of laticifer area in relation to areas of the pericycle and primary phloem (Fig 4b) or secondary phloem (Fig 4d) were similar among the treatments ($p>0.05$).

Discussion

In this study, we investigated the influence of ethylene and jasmonic acid on laticifer differentiation in *T. catharinensis* plants under controlled conditions. We have shown that differentiation of laticifers from ground meristem (in the cortex and pith) is reduced by exogenous ethylene. Concepts of ethylene function in vegetative growth have mainly implicated its potential to both inhibit and stimulate growth in primary tissue (Pierik et al. 2006, Love et al. 2009).

We used ethephon as ethylene-releasing agent. Ethephon is commonly applied in the artificial scrapes on the outer bark of the stem and its concentration ranged from 2.5 at 10% depending of the age and clonal type in cultivation of *H. brasiliensis* for stimulation of latex flow (Moraes and Moraes 2001). The exogenous ethylene leads to

recruitment of isoforms of sucrose transporters (HbSUT1A and HbSUT2A) in the plasma membrane of laticifers (Dusotoit-Coucaud et al. 2009). Such carriers import sucrose into the cytoplasm of the laticifers contributing to increased production of latex in two ways: high rubber synthesis (increased profitability of latex) and, together with polyols and potassium, sucrose to increase the pressure turgor (important for the latex flow) (Dusotoit-Coucaud et al. 2009).

The ethephon concentration used here (2%) reduces the laticifer differentiation in parenchyma tissues in *T. catharinensis*. We highlight that the measurements were made in internodes of the resprouts with usual development. This concentration does not influence the laticifer differentiation in *H. brasiliensis* (Hao & Wu 2000). We did not find reports about the exogenous ethylene effect on reduction of specific tissue or structure, as laticifers. Our findings open a new way to investigation of ethylene effects. The physiological mechanisms associated with this ethylene reduction on laticifer differentiation should involve a complex regulatory network; however, we did not find explanations about this topic.

The effect on defense responses and herbivore resistance in latex-bearing plants are evaluated by transcript levels (Dusotoit-Coucaud et al. 2009, 2010), latex content and amount of latex exudation (Rasman et al. 2009, Agrawal et al. 2014). Although we have not measured the amount of latex exudation, during collection of the basal region of the stem axis we observed that individuals treated with 2% ethephon exude latex in lesser amounts and by shorter time than individuals treated with 0.1% jasmonic acid or deionized water, an indicative of the exogenous ethylene influence latex exudation from secondary phloem. It is important emphasize that the number and proportion of area of laticifers in relation to area of secondary phloem did not differ among the treatments in *T. catharinensis*.

Jasmonic acid appears not to influence on the laticifer differentiation in *T. catharinensis*; although 0.1% jasmonic acid and other lower concentrations (applied in the artificial scrapes on the outer bark) induced the increase in the number of laticifers in the cortex and secondary phloem in *H. brasilienses* (Hao and Wu 2000). In *Asclepias fascicularis*, an Apocynaceae species, jasmonic acid (sprayed, 1.5 ml of 0.5 mM) increase in the amount of latex exudation (Rasman et al. 2009). Jasmonic acid have been implicated in stress-induced responses (Rasman et al. 2009, Agrawal et al. 2014) and, in other hand, they can reduce both cell division and cell expansion (Noir et al. 2013).

Our results differ from those reported in the literature on the influence of ethylene and jasmonic acid on the differentiation of secretory structures, mainly traumatic resin ducts and laticifers. These unexpected results may be related with different causes, e.g. the limited number of surveyed species, developmental stage of the plant, growing conditions, application method, dosage of regulators, among other factors. In addition, even close plant relatives can show qualitatively divergent induced resistance (Agrawal et al. 2014). It is plausible to think that the herbivory do not increase the endogenous ethylene in *T. catharinensis*. Therefore, the laticifer differentiation would not be affected.

Considering the protective latex functions, we suggest that the negative effect of exogenous ethylene on differentiation of laticifers from ground meristem in *T. catharinensis* would make the plants most vulnerable to attack by herbivores and pathogens, mainly shoot apices and young leaves.

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Table, Figures and Figure Captions

Table 1. Analyses of the number of laticifers and proportion of laticifer area in relation to the tissue in the stem of *Tabernaemontana catharinensis* under different plant growth regulators. Measurements represent the mean $\pm$ standard deviation from transverse sections of eleven individuals compared using Tukey's test. Different letters in a same line indicate significant differences. (*) $p < 0.05$; (***) $p < 0.001$.

	Number of laticifers in the stem tissues			(ANOVA)	
	2% ethephon	0.1% jasmonic acid	Water	F _(2,9)	
Cortical parenchyma (0.2mm ²)	15.89 $\pm$ 7.69 a	23.73 $\pm$ 8.16 b	22.09 $\pm$ 5.49 ab	3.6296*	
Pericycle and Primary phloem (0.1mm ²)	19.40 $\pm$ 11.47 a	17.22 $\pm$ 7.36 a	17.86 $\pm$ 8.77 a	0.1101	
Intraxylary phloem and Medullary parenchyma (0.2mm ²)	20.60 $\pm$ 12.00 a	28.06 $\pm$ 11.98 ab	35.13 $\pm$ 11.57 b	4.1314*	
Secondary phloem (0.3mm ²)	23.06 $\pm$ 6.52 a	22.89 $\pm$ 7.75 a	23.94 $\pm$ 6.73 a	0.071	
	Proportion (%) of laticifer area in relation to the tissue in the stem			(ANOVA)	
	2% ethephon	0.1% jasmonic acid	Water	F _(2,9)	
Cortical parenchyma	3.96 $\pm$ 2.63 a	4.79 $\pm$ 1.89 a	4.54 $\pm$ 1.97 a	0.4211	
Pericycle and Primary phloem	6.46 $\pm$ 4.16 a	6.97 $\pm$ 3.21 a	7.09 $\pm$ 2.81 a	0.3243	
Intraxylary phloem and Medullary parenchyma	3.76 $\pm$ 1.96 a	8.50 $\pm$ 3.76 b	9.36 $\pm$ 3.165 b	10.739***	
Secondary phloem	3.98 $\pm$ 1.29 a	3.43 $\pm$ 1.50 a	3.05 $\pm$ 0.84 a	1.5529	

Fig 1. Effects of regulators on plant development in *Tabernaemontana catharinensis*.
a-e. Plants treated with 2% ethephon. **a.** Two days after application. Leaves with necrotic spots. **b-c.** Yellow leaves and fallen leaves. **d.** Cracks in the stem bark. **e.** Thirty days after application. Resprouts next the basal portion of the stem. **f.** Seven days after application of 0.1% jasmonic acid. Plant exhibit usual development.



Fig 2. Internode subjacent to the shoot apex. **a-c, f, g.** Tranverse sections. **d, e.** Longitudinal sections. **a.** Laticifers (*arrows*) in parenchyma tissues (cortical and medullary), in the external and intraxylary phloems. **b.** Laticifers (*arrows*) with different diameters in the cortex. **c-e.** Laticifers in the pith. Adjacent axial chains of laticifers (**c**) and signals of fusion between adjacent chains (**d**). **f-g.** Fusion between laticifers of adjacent chains in the primary phloem (**f**) and pith (**g**).

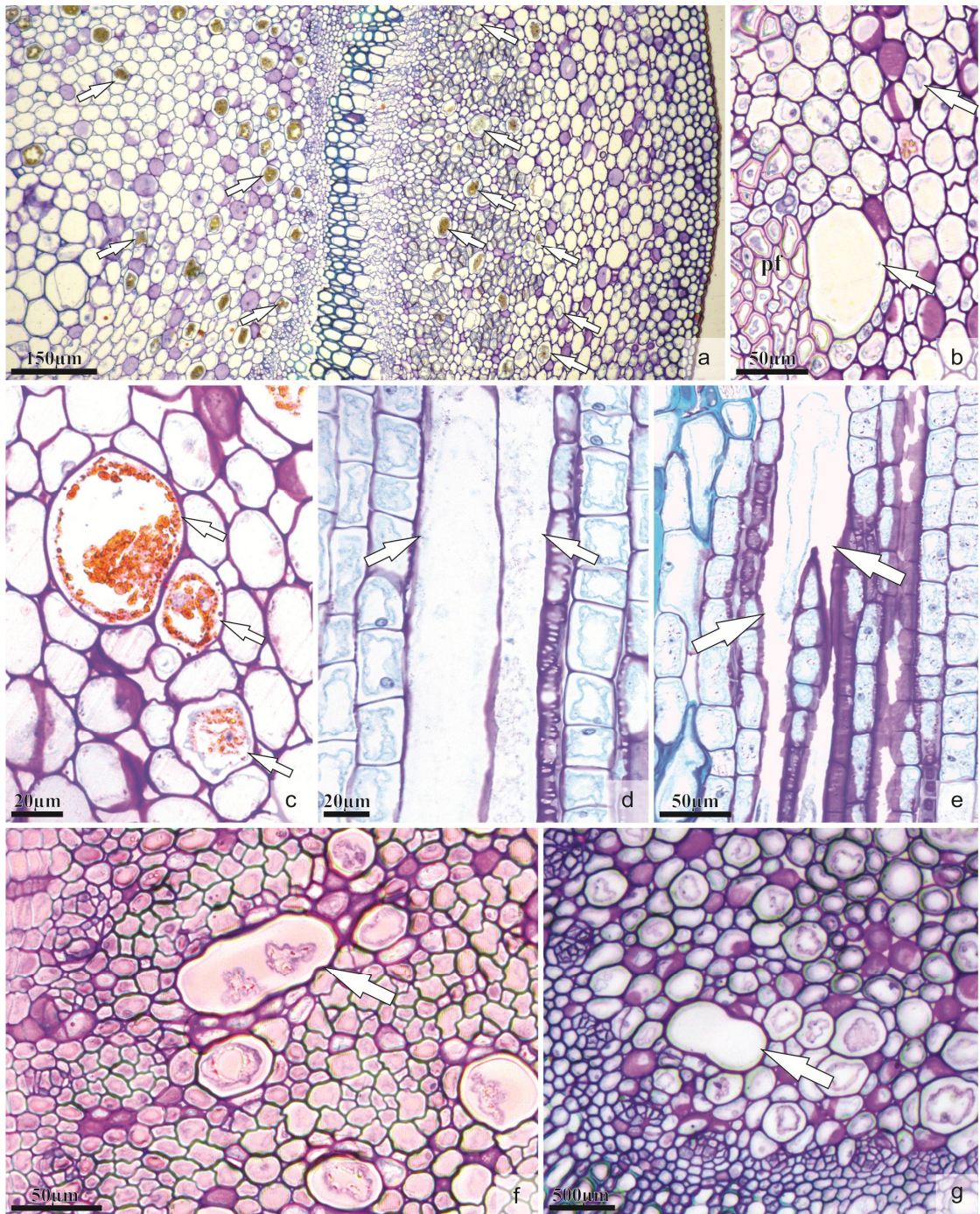


Fig 3. Basal portion of the stem axis. **a-b.** Transverse sections. **c.** Longitudinal section. **a.** Bark comprises the periderm, cortex, pericyclic fibers (*pf*), collapsed primary phloem and secondary phloem (*sp*). **b-c.** Laticifers (*arrows*) in the secondary phloem. *cz* = cambial zone; *ra* = ray; *asterisk* = sieve-tube element.

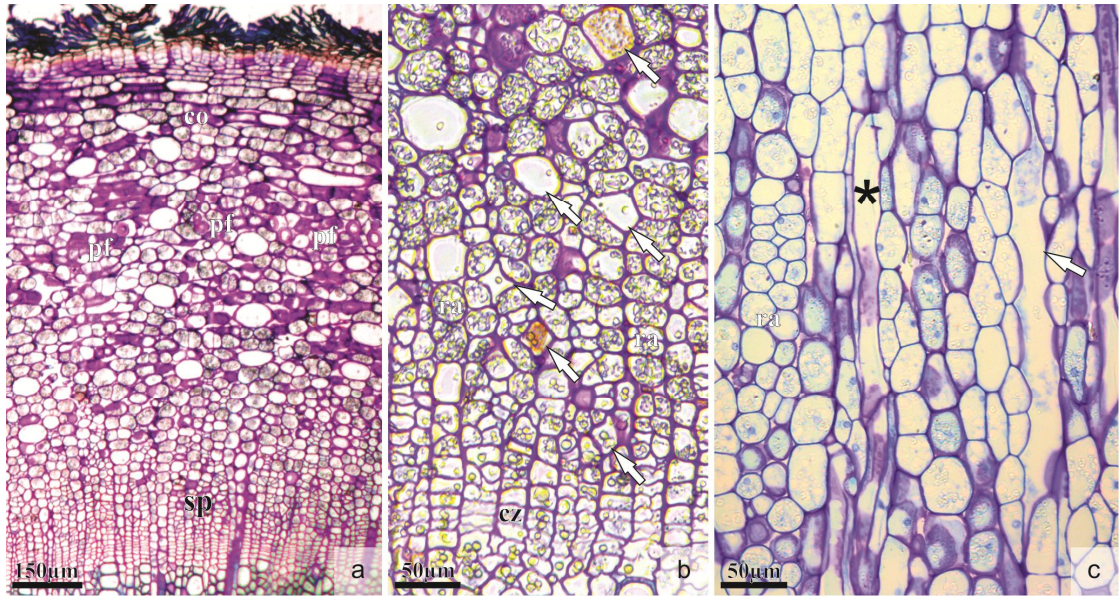
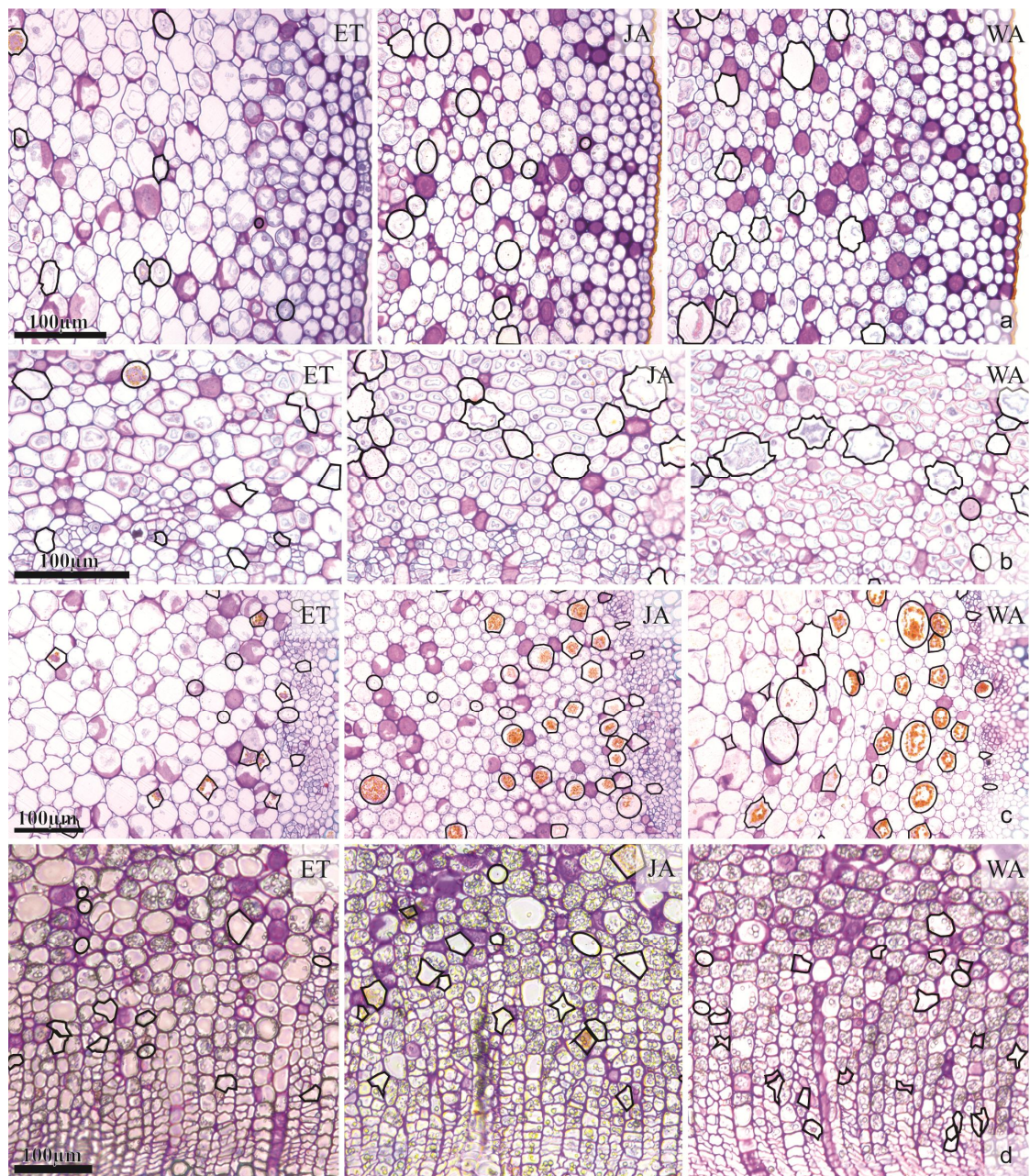


Fig 4. Influence of plant regulators on laticifers of the primary and secondary plant bodies. Transverse sections of the internode subjacent to the shoot apex (**a-c**) and of the basal portion of the stem axis (**d**). Comparison of the laticifers (*outlined in black*) in cortical parenchyma (**a**), pericycle and primary phloem (**b**), intraxylary phloem and medullary parenchyma (**c**) and secondary phloem (**d**).



Considerações gerais

Mais de 20.000 espécies distribuídas em mais de 40 famílias de Angiospermas (8,9% das Angiospermas) exudam látex. O látex da maioria das espécies contém uma variedade de compostos biologicamente ativos, como terpenóides, alcalóides, borracha, proteases, quitinases e glicosidades. Os laticíferos, células que produzem e armazenam látex, constituem importante objeto de estudo; entretanto, os estudos são restritos a poucas espécies de interesse econômico.

Neste estudo, buscamos compreender aspectos relacionados à diferenciação dos laticíferos articulados em *Tabernaemontana catharinensis* A.DC., uma Apocynaceae nativa e amplamente distribuída no Brasil, portadora de um sistema laticífero complexo cujo desenvolvimento envolve a fusão de protoplastos, adição de células e crescimento intrusivo.

As semelhanças na composição de glicanos da parede celular dos laticíferos no ápice caulinar da planta entre espécies portadoras de laticíferos articulados e não articulados com crescimento intrusivo revela que mecanismos envolvidos neste tipo de crescimento são independentes do tipo de laticífero. Uma abordagem interessante para estudos futuros é a verificação destes glicanos de parede em espécies de Apocynaceae com laticíferos articulados, mas que não apresentam crescimento intrusivo.

A semelhança nas características das paredes dos laticíferos e de células meristemáticas no embrião é um indício de que, embora os laticíferos no embrião maduro sejam considerados ativos em secreção, suas paredes ainda podem estar num estado “imaturo”. Nosso estudo é o primeiro a comparar a composição de glicanos de parede celular em laticíferos no embrião e na planta. Assim, estudos que aprofundem

estas comparações para outras espécies vegetais podem ser promissores e importantes para a compreensão da dinâmica da parede celular.

A ação indutora dos laticíferos, levando à aquisição de características de laticíferos por células adjacentes, meristemáticas ou parenquimáticas, foi suportada neste estudo e ocorre por mecanismos distintos no embrião maduro e na planta. No embrião maduro, o crescimento dos laticíferos parece ocorrer predominantemente pela fusão de protoplastos e ação indutora dos laticíferos sobre células adjacentes. O predomínio destes mecanismos de desenvolvimento pode estar relacionado às baixas taxas de multiplicação e alongamento celular no embrião maduro, o que restringiria a expansão dos laticíferos por crescimento intrusivo.

Os resultados obtidos com a aplicação de reguladores vegetais divergiram daqueles encontrados na literatura. O etileno exógeno diminuiu a diferenciação de laticíferos originados do meristema fundamental, enquanto o ácido jasmônico não influenciou a diferenciação dos laticíferos. A influência negativa do etileno exógeno pode ser consequência da concentração utilizada, idade da planta, dentre outros fatores. A indução na diferenciação dos laticíferos pode estar relacionada a outros reguladores vegetais, como por exemplo, o ácido salicílico, um hormônio vegetal também relacionado à resposta a resistência induzida, ou mesmo a uma combinação de reguladores e sinalizadores celulares. Neste contexto, experimentos laboratoriais simulando a herbivoria (e.g. remoção das folhas) ou mesmo a interação da planta com o herbívoro e a imediata avaliação dos níveis de hormônios (etileno, ácido jasmônico, ácido salicílico, ácido abscísico, entre outros) no látex podem contribuir para a escolha das dosagens e hormônios que possam induzir a diferenciação dos laticíferos.

Embora tenhamos encontrado alguns problemas técnicos durante o cultivo de *T. catharinensis* em condições controladas, esta espécie não requer cuidados especiais, a germinação das sementes e a obtenção de plântulas seguem protocolos rotineiros e as plantas podem ser mantidas por períodos longos (cerca de dois anos) em vasos. Estas características favorecem a sua utilização em experimentos laboratoriais. Além disso, o modo de desenvolvimento do seu sistema laticífero a torna ainda mais interessante como um modelo de planta portadora de látex para ser estudada.

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