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ALINE BERTOLOSI BOMBO CARDOSO

ANATOMY AND PHYTOCHEMISTRY OF *Aldama* LA LLAVESPECIES (HELIANTHEAE – ASTERACEAE)

ANATOMIA E FITOQUÍMICA DE ESPÉCIES DE *Aldama* LA LLAVE (HELIANTHEAE – ASTERACEAE)

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RESUMO

A família Asteraceae (Compositae) compreende 1620 gêneros e cerca de 23600, é bem representada no Cerrado, com muitas espécies endêmicas. Subdivide-se em 21 tribos, entre elas Heliantheae, à qual pertence o gênero *Aldama* (=*Viguiera*), e como todos os representantes da tribo, seus representantes são bem caracterizados por estruturas morfológicas reprodutivas. No entanto, as delimitações genéricas muitas vezes não estão bem esclarecidas e estudos baseados apenas em características morfológicas externas são insuficientes para a resolução desses problemas. No Brasil ocorrem 35 espécies do gênero *Aldama* e 27 são endêmicas e distribuídas predominantemente em regiões de Cerrado. É um gênero com classificação taxonômica complexa e problemas de delimitação, e que já foi revisto em diversos estudos, sendo que esses apontam que as espécies brasileiras do grupo formam um grupo coeso juntamente com outros representantes da América do Sul. As espécies desse estudo foram escolhidas devido à dificuldade de delimitação das mesmas, ao seu potencial resinífero e por serem espécies representativas de grupos morfológicos para a realização de futuras análises filogenéticas, e são elas: *Aldama bakeriana*, *A. discolor*, *A. grandiflora* e *A. squalida*. As quatro espécies são muito semelhantes morfologicamente, sendo diferenciadas apenas por características reprodutivas e estudos da morfologia interna poderão auxiliar na elucidação desses problemas de delimitação das mesmas. Além disso, estudos apontam que a composição química de óleos essenciais, além de auxiliar no levantamento de possíveis compostos bioativos, pode ser útil do ponto de vista taxonômico. Assim, a proposta desse estudo visou fornecer dados anatômicos e químicos que possam auxiliar na taxonomia e filogenia do grupo. Para isso, análises anatômicas usuais, bem como técnicas de fitoquímica e biologia molecular foram empregadas. As análises anatômicas comparativas entre as espécies permitiram a identificação de um conjunto de características para cada uma delas, que pode auxiliar na correta delimitação das mesmas. Alterações anatômicas em função de condições edafo-climáticas também foram avaliadas, e verificou-se que não houve variação significativa entre indivíduos de populações e localidades diferentes, em uma mesma espécie. Além de terem sido identificados os sítios de secreção dos compostos que possuem potencial atividade biológica e, ou farmacológica, os estudos sobre os óleos essenciais revelaram que esses compostos podem servir como marcadores químicos nessas espécies, complementando os dados anatômicos para a delimitação das mesmas. E ainda, uma das estruturas secretoras mais relatadas para o gênero e para a família Asteraceae como um todo, o tricoma capitado glandular, foi descrito detalhadamente quanto à sua ontogenia, perfil metabólico e expressão gênica em uma espécie utilizada como modelo para o gênero.

Palavras-chave: Compositae, estruturas secretoras, óleo essencial, taxonomia

ABSTRACT

Asteracae family (Compositae) comprises 1620 genus and about 23600 species. It is well represented in Cerrado areas, with several endemic species. The family is divided into 21 tribes, including Heliantheae, to which belongs the *Aldama* (= *Viguiera*) genus. Its representatives, as all the other representatives of the tribe, are well characterized by their reproductive morphological features. However, generic delimitations are often not quite defined and studies based only on external morphological characteristics are ineffective for solving taxonomic problems. The Brazilian *Aldama* genus comprises 35 species, 17 of which are endemic and mainly distributed in Cerrado areas. It is a genus that has complex taxonomical classification and problems in establishing general and specific boundaries, and it was already reviewed in several studies, which indicated the Brazilian *Aldama* species form a cohesive group along with other South American representatives. The species of this study were chosen because they are difficult to identify taxonomically, they have resiniferous potential, and they are representative species from key morphological groups in the *Aldama* genus and could help in future taxonomical investigations, and they are: *Aldama bakeriana*, *A. discolor*, *A. grandiflora* and *A. squalida*. These four species are morphologically very similar, being differentiated based only on reproductive characteristics and anatomical studies could help in elucidating these delimitation problems. Furthermore, studies have been demonstrated that identifying the chemical composition of these oils can help in the search for possible bioactive compounds and provide useful information for taxonomic studies into the genus. Usual histological techniques, as well as phytochemical and molecular biology techniques, were employed. The comparative analyses among the species allowed the identification of a set of anatomical features for each species, which can help solve the taxonomic problems raised by the four species analyzed herein. Anatomical changed due to the climate and soil conditions were also evaluated, and no significant variation was identified among the different populations and locations analyzed. In addition to the identification of the secretory sites of the compounds having biological and, or pharmacological potential, the studies regarding the essential oils revealed that these compounds can serve as chemical markers in these species, complementing the anatomical data. Also, one of the secretory structures more reported to the genus and to the Asteraceae family as a whole, the capitate glandular trichome, was detailed described in one given species, regarding its ontogeny, metabolic profiles, and gene expression, as a model for the genus.

Key-words: Compositae, essential oil, secretory structures, taxonomy

SUMÁRIO

INTRODUÇÃO

A família Asteraceae Bercht. & J.Presl (Compositae) é uma das maiores famílias entre entre as angiospermas com 1620 gêneros e cerca de 23600 espécies (APG III 2009). Possui distribuição cosmopolita, sendo encontrada em todos os ambientes, exceto o Antártico (Funk et al. 2009) e com centro de origem na América do Sul (Panero e Funk 2008). Seus representantes possuem hábitos variados, podendo ser ervas, subarbustos, árvores e raramente epífitas e lianas (Bremer 1994; Magenta 2006). O sucesso da família na ocupação de tantos ambientes é frequentemente relacionado à ampla gama de metabólitos secundários, à morfologia da inflorescência e eficientes mecanismos de dispersão e à variabilidade morfológica encontrada em seus representantes (Stuessy e Garver 1996; Calabria et al. 2007; Panero e Funk 2008).

Trata-se de uma família monofilética (Funk et al. 2009), caracterizada por flores arranjadas em um receptáculo e cercadas por brácteas constituindo os capítulos, e também pela presença de cipselas frequentemente portadoras de pápus (Bremer 1994; Funk et al. 2009). Apesar de revisões taxonômicas serem frequentes para a família, as relações filogenéticas dentro desta não estão bem estabelecidas (Funk et al. 2009) e ainda há muito a ser realizado, principalmente com as espécies brasileiras.

É uma família bem representada no Cerrado, com muitas espécies endêmicas (Almeida et al. 2005). No entanto, essas são regiões continuamente ameaçadas pela expansão da agropecuária e atividade mineradora (Nakajima e Semir 2001) e estudos com a flora dessas localidades são extremamente importantes, antes que sua diversidade vegetacional seja perdida.

Asteraceae subdivide-se em 12 subfamílias, entre elas Asteroideae, a qual inclui a tribo Heliantheae (Bremer 1994; Panero e Funk 2008; APG III 2009), a segunda maior tribo da família (Spring e Buschman 1996), que apresenta 35 subtribos com 189 gêneros e aproximadamente 2500 espécies (Funk et al. 2009). Seus representantes são predominantemente americanos (Funk et al. 2009), com maior centro de diversificação no México central (Cronquist 1977; Magenta 2006; Baldwin 2009). Representantes dessa tribo são cosmopolitas, principalmente tropicais e subtropicais (Heywood et al. 1977) e são amplamente conhecidos por sua importância econômica, na alimentação com o girassol (*Helianthus annuus* L.) (Souza e Lorenzi 2008) e o yacón (*Smallanthus sonchifolius* (Poepp.) H.Rob. (Figueiredo-Ribeiro et al. 1992; Fukai et al. 1993) e também por sua utilização na

medicina popular, com a equinácea (*Echinacea angustifolia* DC.) (Silveira et al. 2008) e o jambú (*Spilanthes oleracea* L.) (Martins et al. 2012).

A tribo Heliantheae, bem como todos os representantes da subtribo Helianthinae, a qual pertence o gênero *Aldama* La Llave (=*Viguiera* Kunth), são bem caracterizados por atributos morfológicos reprodutivos (Robinson e Moore 2004; Magenta 2006). Delimitações dos gêneros, no entanto, muitas vezes não são bem definidas e Schilling e Panero, já em 1996, destacaram a ineficácia dos estudos com base apenas nas características morfológicas externas para resolver problemas taxonômicos.

Das 35 espécies do gênero *Aldama* que ocorrem no Brasil, 27 são endêmicas e distribuídas predominantemente em regiões de Cerrado (Magenta et al. 2010). O gênero se caracteriza por representantes perenes, sendo ervas anuais ou arbustos, com folhas opostas ou alternas, simples ou algumas vezes, pinatissectas com capítulos corimbiformes, paniculados ou solitários, radiados, envolvidos por brácteas involucrais em duas a sete camadas. As flores do raio podem ser femininas ou neutras, amarelas, cremes ou laranjas; as flores do disco geralmente são amarelas. Os frutos são cipselas obovada-oblongas coroadas por pápus com duas aristas intercaladas por escamas paleáceas (Bremer 1994).

Trata-se de um gênero com classificação taxonômica complexa e problemas de delimitação, e que já foi revisto por diversos autores (Robinson 1977, 1981; Panero e Schilling 1988; Schilling e Jansen 1989; Schilling e Panero 1996; Schilling et al. 2000; Magenta 2006; Magenta et al. 2010). Tradicionalmente reconhecido dentro do gênero *Viguiera* Kunth (Blake 1918), o grupo de espécies brasileiras, juntamente com todas as espécies de *Viguiera* da América do Sul, foi transferido para *Aldama* La Llave por Schilling e Panero (2011), com base em análises moleculares de ITS, ETS e cpDNA. Dessa forma, as espécies brasileiras do grupo foram sinonimizadas para o gênero *Aldama*. Fica claro que as espécies brasileiras formam um grupo coeso juntamente com outros representantes da América do Sul. No entanto, problemas em delimitações genéricas e específicas prevalecem entre representantes do gênero (Magenta et al. 2010).

A maioria dos trabalhos sobre o gênero *Aldama* (= *Viguiera* Kunth) está relacionada à fitoquímica e atividade farmacológica de extratos (Da Costa et al. 1998; Marquina et al. 2001; Spring et al. 2003; Ambrosio et al. 2004; Arakawa et al. 2008; Nicolete et al. 2009; Carvalho et al. 2011). A atividade farmacológica das plantas desse gênero é atribuída, geralmente, às lactonas sesquiterpênicas (Da Costa et al. 2005), substâncias utilizadas no tratamento de doenças inflamatórias na medicina tradicional (Siedle et al. 2004). Esses compostos apresentam uma variedade de atividades tais como antibacteriana em

Viguiera dentata (Canales et al. 2008), antimicrobiana em *A. arenaria* (Porto et al. 2009), antiinflamatória, analgésica (Valério et al. 2007) e citotóxica (Arakawa et al. 2008) em *A. robusta*. Além disso, estudos mais recentes têm demonstrado que a composição química dos óleos essenciais em espécies de *Aldama* pode auxiliar no levantamento de possíveis compostos bioativos (Oliveira et al. 2011; Rehder et al. 2012).

As espécies desse estudo são *Aldama bakeriana* S.F. Blake E.E.Schill. & Panero, *A. discolor* (Baker) E.E.Schill. & Panero, *A. grandiflora* (Gardner) E.E.Schill. & Panero e *A. squalida* (S. Moore) E.E.Schill. & Panero e foram escolhidas devido à dificuldade de delimitação das mesmas, ao seu potencial resinífero e por serem espécies representativas de grupos morfológicos para a realização de futuras análises filogenéticas, dentro do Projeto Temático "Estudos morfoanatômicos, metabolômicos e moleculares como subsídios à sistemática de espécies de Asteraceae e acesso ao seu potencial farmacológico" (Processo FAPESP 2010/51454-3).

De acordo com a classificação artificial proposta por Blake (1918) e atualizada por Magenta (2006) essas espécies pertencem à Seção Paradosa, série Grandiflorae, e das 11 espécies dessa série, as quatro abordadas nesse estudo são endêmicas do Brasil. Se caracterizam por ervas perenes, com folhas inferiores opostas e frequentemente escamiformes e espessadas, e as superiores mais espessadas, maiores e alternas, fortemente nervuradas, com 5 a 7 nervuras principais; os capítulos são solitários ou poucos, grandes, terminais, com brácteas involucrais em 2-4 séries, de tamanhos semelhantes, lanceoladas ou oblanceoladas, pouco endurecidas.

Segundo Magenta et al. (2010), é possível que *Aldama bakeriana*, *A. discolor* e *A. squalida* sejam uma única espécie, sendo que *A. discolor* diferencia-se de *A. bakeriana* apenas por características das brácteas involucrais externas dos capítulos e dos pápus das cipselas. *A. discolor* também é semelhante à *A. squalida* em relação ao hábito e à morfologia das cipselas. Já *A. grandiflora* foi incluída nas análises, pois se trata da espécie tipo da seção Grandiflorae e, além disso, *A. grandiflora* e *A. squalida* apresentam indivíduos muito semelhantes em estado vegetativo, sendo às vezes impossível diferenciá-los (Magenta et al. 2010). Dessa forma, uma vez que apenas características reprodutivas permitem fazer essa distinção (Magenta et al. 2010), estudos da morfologia interna podem auxiliar na elucidação desses problemas de delimitação das espécies.

As análises anatômicas têm representado uma eficiente ferramenta em estudos taxonômicos da família Asteraceae (Adedeji e Jewolla 2008; Duarte et al. 2008; Cury e Appezzato-da-Glória 2009; Inceer e Ozcan 2011; Sosa et al. 2013), inclusive para o gênero

Aldama (Castro et al. 1997; Bombo et al. 2012; Oliveira et al. 2013; Bombo et al. 2014; Silva et al. 2014), principalmente em relação às estruturas secretoras, que possuem grande valor diagnóstico, pois variam quanto à sua anatomia e também apresentam considerável constância quanto à posição em que ocorrem nas espécies (Solereder 1908; Metcalfe e Chalk 1950; Castro et al. 1997; Fahn 2000).

A presença das estruturas secretoras em espécies da família Asteraceae e no gênero *Aldama* está relacionada à presença de compostos do metabolismo secundário (Agostini et al. 2005; Castro et al. 2006; Borsato et al. 2007; Maia et al. 2010; Bombo et al. 2014, 2012), bem como sua potencial atividade biológica e, ou farmacológica (Marquina et al. 2001; Spring et al. 2003; Ambrosio et al. 2004; Valério et al. 2007; Canales et al. 2008; Arakawa et al. 2008; Nicolete et al. 2009; Porto et al. 2009; Carvalho et al. 2011). Já os estudos sobre óleos essenciais no gênero são restritos ao nosso grupo de pesquisa (Appezzato-da-Glória et al. 2012; Bombo et al. 2012; Filartiga et al. 2012; Rehder et al. 2012; Silva et al. 2013; Bombo et al. 2014) e esses têm demonstrado que a composição química dos óleos essenciais pode ser útil do ponto de vista taxonômico (Oliveira 2011; Bombo et al. 2012; Silva 2013), além de auxiliar no levantamento de possíveis compostos bioativos (Appezzato-da-Glória et al. 2012; Rehder et al. 2012; Oliveira et al. 2013).

Diante do exposto, o presente estudo visa fornecer dados anatômicos e químicos de quatro espécies do gênero *Aldama*, que possam auxiliar na taxonomia do gênero.

OBJETIVOS

Os objetivos principais dessa tese foram:

a) Caracterizar a anatomia dos órgãos vegetativos de quatro espécies de *Aldama* e identificar características anatômicas potencialmente úteis para resolver problemas taxonômicos do grupo;

b) Avaliar quantitativamente a morfoanatomia de uma das espécies, provenientes de seis populações diferentes, para avaliar a influência da distribuição geográfica e dos fatores edafo-climáticos nas características anatômicas;

c) Caracterizar os óleos essenciais dos órgãos vegetativos dessas espécies e identificar seus sítios de secreção e armazenamento;

d) Aprofundar o conhecimento sobre o tricoma capitado glandular em espécies de *Aldama*, caracterizando o desenvolvimento, morfologia e atividade metabólica do mesmo em *A. discolor*, como um modelo para o gênero.

ESTRUTURAÇÃO DA TESE

A tese foi organizada na forma de capítulos, sendo que cada um deles aborda um aspecto diferente da pesquisa. Os estudos anatômicos conduzidos com as espécies de *Aldama* selecionadas para essa tese foram realizados com indivíduos provenientes de populações diferentes para cada uma das espécies, com a finalidade de abranger a maior parte das variações anatômicas e dessa forma, caracterizar com maior precisão a anatomia das mesmas. A partir dos resultados, um conjunto de características anatômicas dos órgãos vegetativos foi elaborado para cada espécie (capítulo 1), o qual permitiu a diferenciação das mesmas.

No decorrer dos estudos anatômicos, observou-se que a anatomia da espécie *Aldama grandiflora* apresentava pequenas variações entre indivíduos de uma mesma população e entre as populações de localidades diferentes. Essas observações conduziram a um estudo que visou quantificar as características micromorfológicas das folhas e caules, e avaliar se as condições sob as quais essas populações se encontravam poderiam estar influenciando na micromorfologia de seus indivíduos (Capítulo 2).

Como já descrito anteriormente, diversas espécies de *Aldama* são potencialmente resiníferas e produtoras de óleos essenciais (Magenta et al. 2010). Uma vez que a composição química destes óleos tem auxiliado na busca de possíveis compostos bioativos (Appezzato-da-Glória et al. 2012; Rehder et al. 2012; Oliveira et al. 2013), e fornecido informações úteis para estudos taxonômicos (Oliveira 2011; Bombo et al. 2012; Silva 2013, Bombo et al. 2014), as quatro espécies também foram avaliadas quanto ao seu rendimento e composição química dos óleos essenciais dos órgãos vegetativos (Capítulo 3). Também foi descrita a ocorrência e o posicionamento das estruturas secretoras relacionadas à produção destes metabólitos no corpo da planta, uma vez que esses dados podem servir de base para futuros estudos sobre a atividade biológica e farmacológica, indicando quais órgãos merecem uma investigação mais aprofundada.

Entre as estruturas secretoras descritas para as quatro espécies aqui analisadas e também em outras *Aldama* (Bombo et al. 2012; Oliveira et al. 2013; Silva et al. 2014), o tricoma glandular capitado tem grande destaque, pois é de ampla ocorrência na família

Asteraceae (Werker e Fahn 1981; Castro et al. 1997; Monteiro et al. 2001; Mayekiso et al. 2008; Amrehn et al. 2014) e tem sido bastante estudado devido à produção de diversos metabólitos, tais como terpenoides e flavonoides, com destaque para as lactonas sesquiterpênicas (Kelsey et al. 1984; Duke et al. 2000; Göpfert et al. 2009;). Apesar de a morfologia e química desses tricomas ser bem conhecida, os genes envolvidos na biossíntese dessas lactonas têm sido identificados recentemente e caracterizados apenas em *Helianthus annuus* (Göpfert 2008; Göpfert et al. 2009; Nguyen et al. 2010; Ikezawa et al. 2011) e para espécies brasileiras esses aspectos nunca foram abordados. Nesse contexto, o capítulo 4 descreve os resultados obtidos durante o doutorado sanduíche, realizado em parceria com o grupo de pesquisa que vem estudando esses aspectos nos tricomas de *H. annuus* (Göpfert et al. 2005; Amrehn et al. 2014; Aschenbrenner et al. 2015). A espécie *Aldama discolor* foi utilizada como modelo e detalhes da morfologia, ontogênese, perfil metabólico e expressão gênica relacionados à esse tricoma foram abordados.

REFERÊNCIAS BIBLIOGRÁFICAS

ADEDEJI, O.; JEWOOLA, O. 2008. Importance of Leaf Epidermal Characters in the Asteraceae Family. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 36: 7-16.

AGOSTINI, F.; SANTOS, A.C.A.; ROSSATO, M.; PANSERA, M.R.; ZATTERA, F.; WASUM, R.; SERAFINI, L.A. 2005. Estudo do óleo essencial de algumas espécies do gênero *Baccharis* (Asteraceae) do sul do Brasil. Revista Brasileira de Farmacognosia 15: 215-220.

ALMEIDA, A.M.D.; FONSECA, C.R.; PRADO, P.I.; ALMEIDA-NETO, M.; DINIZ, S.; KUBOTA, U.; BRAUN, M.R. et al. 2005. Diversidade e ocorrência de asteraceae em cerrados de São Paulo. Biota Neotropica 5: 1-17.

AMBROSIO, S.; SCHORR, K.; DA COSTA, F.B. 2004. Terpenoids of *Viguiera arenaria* (Asteraceae). Biochemical Systematics and Ecology 32: 221-224.

AMREHN, E.; HELLER, A.; SPRING, O. 2014. Capitate glandular trichomes of *Helianthus annuus* (Asteraceae): ultrastructure and cytological development. Protoplasma 251: 161-167.

APG III, ANGIOSPERM PHYLOGENY GROUP. 2009. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III. Botanical Journal of the Linnean Society 161: 105-121.

APPEZZATO-DA-GLÓRIA, B.; BOMBO, A.B.; OLIVEIRA, A.S.S.; RUIZ, A.L.T.G.; CARVALHO, J.E.; REHDER, V. L. G. 2012. Chemical composition, byosinthesis sites and evaluation of *in vitro* antiproliferative activity of essential oils of *Aldama filifolia* (Asteraceae), Annals of 43rd International Symposium on Essential Oils.

ARAKAWA, N.; SCHORR, K.; AMBROSIO, S.; MERFORT, I.; DA COSTA, F. 2008. Further sesquiterpene lactones from *Viguiera robusta* and the potential anti-inflammatory activity of a heliangolide: Inhibition of human neutrophil elastase release. Zeitschrift Fur Naturforschung Section C - A Journal of Biosciences 63: 533-538.

ASCHENBRENNER, A.K.; AMREHN, E.; BECHTEL, L.; SPRING, O. 2015. Trichome differentiation on leaf primordia of *Helianthus annuus* (Asteraceae): morphology, gene expression and metabolite profile. Planta 241: 837-846.

BALDWIN, B.G. 2009. Heliantheae Alliance. *In:*FUNK, V.A.; SUSANNA, A.; STUESSY, T.F.; BAYER, R.J. Eds. Systematics, Evolution, and Biogeography of Compositae. International Association for Plant Taxonomy, Vienna, Austria, pp. 689-711.

BLAKE, S.F. 1918. A revision of the genus *Viguiera*. *C*ontributions from the Gray Herbarium of Harvard University 51: 1-205.

BOMBO, A.; OLIVEIRA, T.S.; OLIVEIRA, A.S.S.; REHDER, V.L.G.;MAGENTA, M.A.G.; APPEZZATO-DA-GLÓRIA, B. 2012. Anatomy and essential oils from aerial organs in three species of *Aldama* (Asteraceae-Heliantheae) that have a difficult delimitation. Australian Journal of Botany 60: 632-642.

BOMBO, A.; OLIVEIRA, T.S.; OLIVEIRA, A.S.S.; REHDER, V.L.G.;APPEZZATO-DA-GLÓRIA, 2014. Anatomy and essential oil composition of the underground systems of three species of *Aldama* La Llave (Asteraceae). Journal of the Torrey Botanical Society 141: 115-125.

BORSATO, A.V.; DONI-FILHO, L.; CÔCCO, L.C.; PAGLIA, E.C. 2007. Rendimento e composição química do óleo essencial da camomila [*Chamomilla recutita* (L.) Rauschert] submetida à secagem à 70º C. Ciências Agrárias 28: 635-644.

BREMER, K. 1994. Asteraceae: cladistics and classification. Portland: Timber Press, 752p.

CALABRIA, L.; EMERENCIANO, V.; FERREIRA, M.; SCOTTI, M.; MABRY, T. 2007. A phylogenetic analysis of tribes of the Asteraceae based on phytochemical data. Natural Product Communications 2: 277-285.

CANALES, M.; HERNÁNDEZ, T.; RODRÍGUEZ-MONROY, M.A.; JIMÉNEZ-ESTRADA, M.; FLORES, C.M.; HERNÁNDEZ, L.B.,; GIJÓN, I.C.; et al. 2008. Antimicrobial Activity of the Extracts and Essential Oil of *Viguiera dentata*. Pharmaceutical Biology 46: 719-723.

CARVALHO, T.; SIMAO, M.; AMBROSIO, S.; FURTADO, N.; VENEZIANI, R.; HELENO, V.; DA COSTA, F.B.; et al. 2011. Antimicrobial Activity of Diterpenes from *Viguiera arenaria* against Endodontic Bacteria. Molecules 16: 543-551.

CASTRO, D.P.; CARDOSO, M.G.; MORAES, J.C.; SANTOS, N.M.; BALIZA, D.P. 2006. Não-preferência de *Spodoptera frugiperda* (Lepidoptera: Noctuidae) por óleos essenciais de *Achillea millefolium* L. e *Thymus vulgaris* L. Revista Brasileira de Plantas Medicinais 8: 27-32.

CASTRO, M.D.M.; LEITÃO-FILHO, H.D.F.; MONTEIRO, W.R. 1997. Utilização de estruturas secretoras na identificação dos gêneros de Asteraceae de uma vegetação de cerrado. Revista Brasileira de Botânica 20: 163-174.

CRONQUIST, A. 1977. The Compositae revisited. Brittonia 29: 137-153.

CURY, G.; APPEZZATO-DA-GLÓRIA, B. 2009. Internal secretory spaces in thickened underground systems of Asteraceae species. Australian Journal of Botany 57: 229-239.

DA COSTA, F.B.; ITO, L.; ANDRE, R.; VICHNEWSKI, W.; HERZ, W. 1998. Constituents of *Viguiera* species with antibacterial activity. Fitoterapia 69: 86-87.

DA COSTA, F.B.; TERFLOTH, L.; GASTEIGER, J. 2005. Sesquiterpene lactone-based classification of three Asteraceae tribes: a study based on self-organizing neural networks applied to chemosystematics. Phytochemistry 66: 345-353.

DUARTE, M.; WOLF, S.; PAULA, B. 2008. *Smallanthus sonchifolius* (Poepp.) H. Rob. (yacon): microscopical identification of the leaf and stem for the pharmacognostic quality control. Revista Brasileira De Ciencias Farmaceuticas 44: 157-164.

DUKE, S.O.; CANEL, C.; RIMANDO, A.M.; TELLEZ, M. R.; DUKE, M.V.; PAUL, R.N. 2000. Current and potential exploitation of plant glandular trichome productivity. *In:*HALLAHAN, D.L.; GRAY J.C. Eds., Advances in Botanical Research 31: 121-151. Academic Press, San Diego.

FAHN, A. 2000. Structure and function of secretory cells. Advances in Botanical Research Incorporating Advances in Plant Pathology 31: 37-75.

FIGUEIREDO-RIBEIRO, R.C.; DIETRICH, S.M.C.; CARVALHO, M.A.M.; VIEIRA, C.C.J.; ISEJIMA, E.M.; DIAS-TAGLIACOZZO, G.M.; TERTULIANO, M.F. 1992. As múltiplas utilidades dos frutanos: reserva de carboidratos em plantas nativas do cerrado. Ciência Hoje 14: 16-18

FILARTIGA, A.L.P.; FARIAS, L.O.; REHDER, V.L.G.; APPEZZATO-DA-GLÓRIA, B. 2012. Anatomia e composição química do óleo essencial da folha de *Viguiera megapotamica* Malme (Asteraceae), 63º Congresso Nacional de Botânica. Anais do 63º Congresso Nacional de Botânica., Joinville, SC, Brasil.

FUKAI, K.; MIYAZAKI, S.; NANJO, F.; HARA, Y. 1993. Distribution of carbohydrates and related enzymes activities in yacon (*Polymnia sonchifolia*). Soil Science and Plant Nutrition 39: 567-571.

FUNK, V.A.; SUSANNA, A.; STUESSY, T.F.; BAYER, R.J. 2009. Systematics, Evolution, and Biogeography of Compositae. International Association for Plant Taxonomy, Vienna, Austria.

GÖPFERT, J.; HEIL, N.; CONRAD, J.; SPRING, O. 2005. Cytological development and sesquiterpene lactone secretion in capitate glandular trichomes of sunflower. Plant Biology 7: 148-155.

GÖPFERT, J.C. 2008. Molekulare und entwicklungsbiologische Charakterisierung von Schlüsselenzymen der Naturstoffbiosynthese in Drüsenhaaren der Sonnenblume Doktorgrades der Naturwissenschaften Universität Hohenheim, Sindelfingen.

GÖPFERT, J.C.; MACNEVIN, G.; RO, D.K.; SPRING, O. 2009. Identification, functional characterization and developmental regulation of sesquiterpene synthases from sunflower capitate glandular trichomes. BMC Plant Biology 9: 86.

HEYWOOD, V.H.; HARBONE, J.B.; TURNER, B.L. 1977. The biology and chemistry of the Compositae. 2 vols. Academic Press. New York.

IKEZAWA, N.; GÖPFERT, J.C.; NGUYEN, D.T.; KIM, S.U.; O'MAILLE, P.E.; SPRING, O.; RO, D.K. 2011. Lettuce costunolide synthase (CYP71BL2) and its homolog (CYP71BL1) from sunflower catalyze distinct regio- and stereoselective hydroxylations in sesquiterpene lactone metabolism. Journal of Biological Chemistry 286: 21601-21611.

INCEER, H.; OZCAN, M. 2011. Leaf anatomy as an additional taxonomy tool for 18 taxa of *Matricaria* L. and *Tripleurospermum* Sch Bip. (Anthemideae-Asteraceae) in Turkey. Plant Systematics and Evolution 296: 205-215.

KELSEY, R. 1984. Glandular trichomes - a helpful taxonomic character for *Artemisia nova* (black sagebrush). Journal of Range Management 37: 370-372.

MAGENTA, M.A.G. 2006. *Viguiera* Kunth (Asteraceae, Heliantheae) na América do Sul e sistemática das espécies do Brasil. doutorado, Universidade de São Paulo, São Paulo, São Paulo.

MAGENTA, M.A.G.; PIRANI, J.R.; MONDIN, C.A. 2010. Novos táxons e combinações em *Viguiera* (Asteraceae - Heliantheae). Rodriguésia 61: 1-11.

MAIA, A.; TORRES, M.; PESSOA, O.; MENEZES, J.; COSTA, S.; NOGUEIRA, V.; MELO, V. et al. 2010. Volatile leaf oils of *Vernonia brasiliano* and *Vernonia remotiflora*: chemical composition and biological activity. Quimica Nova 33: 584-586.

MARQUINA, S.; MALDONADO, N.; GARDUNO-RAMIREZ, M.; ARANDA, E.; VILLARREAL, M.; NAVARRO, V.; BYE, R. et al. 2001. Bioactive oleanolic acid saponins and other constituents from the roots of *Viguiera decurrens*. Phytochemistry 56: 93-97.

MARTINS, C.P.S.; MELO, M.T.P.; HONÓRIO, I.C.G.; D'ÁVILA, V.A.; CARVALHO JÚNIOR, W.G.O. 2012. Caracterização morfológica e agronômica de acessos de jambu (*Spilanthes oleracea* L.) nas condições do Norte de Minas Gerais. Revista Brasileira de Plantas Medicinais 14(2): 410-413

MAYEKISO, B.; MAGWA, M.L.; COOPOOSAMY, R.. 2008. The morphology and ultrastructure of glandular and non-glandular trichomes of *Pteronia incana* (Asteraceae). African Journal of Plant Science 2: 52-60.

METCALFE, C.R.; CHALK, L. 1950. Anatomy of the dicotyledons. Clarendon Press, Oxford.

MONTEIRO, W.R.; CASTRO, M.M.; MAZZONI-VIVEIROS, S.C.; MAHLBERG, P.G. 2001. Development and some histochemical aspects of foliar glandular trichomes of *Stevia rebaudiana* (Bert.) Bert. – Asteraceae. Revista Brasileira de Botânica 24: 349-357.

NAKAJIMA, J.N.; SEMIR, J. 2001. Asteraceae no Parque Nacional da Serra da Canastra, Minas Gerais, Brasil. *Revista Brasileira de Botânica* 24: 471-478.

NGUYEN, D.T.; GÖPFERT, J.C.; IKEZAWA, N.; MACNEVIN, G.; KATHIRESAN, M.; CONRAD, J.; SPRING, O. et al. 2010. Biochemical conservation and evolution of germacrene A oxidase in Asteraceae. Journal of Biological Chemistry 285: 16588-16598.

NICOLETE, R.; ARAKAWA, N.; RIUS, C.; NOMIZO, A.; JOSE, P.; DA COSTA, F.B.; SANZ, M. et al. 2009. Budlein A from *Viguiera robusta* inhibits leukocyte-endothelial cell interactions, adhesion molecule expression and inflammatory mediators release. Phytomedicine 16: 904-915.

OLIVEIRA, A.S.S.; REHDER, V.L.G.; RUIZ, A.L.T.G.; SOUSA, V.H.S.; CARVALHO, J.E.; ALMEIDA, T.S.O.; APPEZZATO-DA-GLÓRIA, B. et al. 2011. Avaliação da atividade anticâncer do extrato clorofórmico das raízes de *Viguiera arenaria* Baker. Reunião Anual da Sociedade Brasileira de Química, Florianópolis.

OLIVEIRA, T.S.D.; BOMBO, A.B.; APPEZZATO-DA-GLÓRIA, B. 2013. Anatomy of vegetative organs with an emphasis on the secretory structures of two species of *Aldama* (Asteraceae – Heliantheae). Botany 91(6): 335-342.

PANERO, J.; FUNK, V. 2008. The value of sampling anomalous taxa in phylogenetic studies: Major clades of the Asteraceae revealed. Molecular Phylogenetics and Evolution 47: 757-782.

PORTO, T.; FURTADO, N.; HELENO, V.; MARTINS, C.; DA COSTA, F.B.; SEVERIANO, M.; SILVA, A. et al. 2009. Antimicrobial ent-pimarane diterpenes from *Viguiera arenaria* against Gram-positive bacteria. Fitoterapia 80: 432-436.

REHDER, V.L.G.; OLIVEIRA, A.S.S.; BOMBO, A.B.; RUIZ, A.L.T.G.; CARVALHO, J.E.; APPEZZATO-DA-GLÓRIA, B. 2012. Evaluation of chemical composition and *in vitro* antiproliferative activity of essential oils of different parts of *Aldama linearifolia* Chodat, 43rd International Symposium on Essential Oils, Lisbon, Portugal.

ROBINSON, H. 1977. Studies in Heliantheae (Asteraceae). VIII. Notes on genus and species limits in the genus *Viguiera*. Phytologia 36: 201-215.

ROBINSON, H. 1981. A revision of the tribal and subtribal limits of the Heliantheae (Asteraceae). Smithson Contribution to Botany 51: 1-102.

ROBINSON, H.; MOORE, A. 2004. New species and new combinations in *Rhysolepis* (Heliantheae : Asteraceae). Proceedings of the Biological Society of Washington 117: 423-446.

SCHILLING, E.; JANSEN, R. 1989. Restriction fragment analysis of chloroplast DNA and the systematics of *Viguiera* and related genera (ASTERACEAE, HELIANTHEAE). American Journal of Botany 76: 1769-1778.

SCHILLING, E.; PANERO, J. 1996. Phylogenetic reticulation in subtribe Helianthinae. American Journal of Botany 83: 939-948.

SCHILLING, E.;PANERO, J. 2011. A revised classification of subtribe Helianthinae (Asteraceae: Heliantheae) II. Derived lineages. Botanical Journal of the Linnean Society 167: 311-331.

SCHILLING, E.E.; DA COSTA, F.B.; LOPES, N.P.; HEISE, P.J. 2000. Brazilian species of *Viguiera* (Asteraceae) exhibit low levels of its sequence variation. Edinburgh Journal of Botany 57: 323-332.

SIEDLE, B.; GARCÍA-PIÑERES, A.J.; MURILLO, R.; SCHULTE-MÖNTING, J.; CASTRO, V.; RÜNGELER, P.; KLAAS, C.A. et al. 2004. Quantitative Structure-Activity Relationship of Sesquiterpene Lactones as Inibitors of the Transcription Factor NF-κB. Journal of Medicinal Chemistry 47: 6042-6054.

SILVA, E.M.S. 2013. Anatomia e análise do óleo essencial dos órgãos vegetativos de *Aldama tenuifolia* (Gardner) E.E.Schill. & Panero e *A. kunthiana* (Gardner) E.E.Schill. & Panero (Asteraceae - Heliantheae). Mestrado, Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Piracicaba, Brasil.

SILVA, E.M.S.D.; HAYASHI, A.H.; APPEZZATO-DA-GLÓRIA, B. 2014. Anatomy of vegetative organs in *Aldama tenuifolia* and *A. kunthiana* (Asteraceae: Heliantheae). *Brazilian Journal of Botany* 37: 505-507.

SILVEIRA, P.F.; BANDEIRA, M.A.M.; ARRAIS, P.S.D. 2008. Farmacovigilância e reações adversas às plantas medicinais e fitoterápicos: uma realidade. Brazilian Journal of Pharmacognosy 18(4): 618-626

SOLEREDER, H. 1908. Systematic anatomy of the dicotyledons: A handbook for laboratories of pure and applied botany. Clarendon Press, Oxford.

SOUZA, V.C.; LORENZI, H. 2005. Botânica sistemática: guia ilustrado para identificação dasfamílias de Angiospermas da flora brasileira baseado em APG II, Nova Odessa: Instituto Plantarum.

SOSA, M. M.; PICO, G.M.V.D.; DEMATTEIS, M. 2013. Comparative anatomy of leaves and stems in some species of the South American genus *Chrysolaena* (Vernonieae, Asteraceae) and taxonomic implications. Nordic Journal of Botany 32: 611-619.

SPRING, O.; BUSCHMANN, H. 1996. A chemotaxonomiv survey of sesquiterpene lactones in the Helianthinae (Compositae). Compositae: Systematics 1: 307-316.

SPRING, O.; ZIPPER, R.; CONRAD, J.; VOGLER, B.; KLAIBER, I.; DA COSTA, F.B. 2003. Sesquiterpene lactones from glandular trichomes of *Viguiera radula* (Heliantheae; Asteraceae). Phytochemistry 62: 1185-1189.

STUESSY, T.F.; GARVER, D. 1996. The defensive role of pappus in heads of Compositae. *In:*CALIGARI, P.D.S.; HIND, D.J.N. Eds., Compositae: biology and utilization. Proceedings of the International Compositae Conference, Kew, Royal Botanic Garden 81-91.

VALERIO, D.; CUNHA, T.; ARAKAWA, N.; LEMOS, H.; DA COSTA, F.B.; PARADA, C.; FERREIRA, S. et al. 2007. Anti-inflammatory and analgesic effects of the sesquiterpene lactone budlein A in mice: Inhibition of cytokine production-dependent mechanism. European Journal of Pharmacology 562: 155-163.

WERKER, E.; FAHN, A. 1981. Secretory Hairs of *Inula viscosa* (L.) Ait.-Development, Ultrastructure, and Secretion. Botanical Gazette 142: 461-476.

CAPÍTULO I

Solving taxonomic problems within the *Aldama* genus based on anatomical characters

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Solving taxonomic problems within the *Aldama* genus based on anatomical characters

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Abstract

Anatomical characteristics have proved to be an invaluable asset for supporting taxonomic studies on different plant families, such as Asteraceae. Anatomical features can also help clarify taxonomical problems in the *Aldama* La Llave genus, especially among Brazilian representatives. The present study focussed on *Aldama bakeriana*, *A. discolor*, *A. grandiflora* and *A. squalida.* These species were chosen because they are difficult to identify taxonomically if the specimens have no flowers, they have biological and pharmacological potential and they are representative species from key morphological groups in the *Aldama* genus and could help in future taxonomical investigations. Aerial and underground vegetative organs from the four species were described herein for the first time and a comparative analysis was performed to highlight the unique features of each species and determine whether these species can be differentiated in terms of anatomy. All four species analysed were anatomically very similar. However, they could be differentiated on the basis of the set of anatomical features described for each species. On the basis of our findings, we concluded that anatomy is able to provide data to assist with the taxonomic problems within the four species analysed herein. The results also corroborated other studies on the *Aldama* genus.

Anatomical characteristics are important for taxonomic studies on different plant families. The present study focussed on four Brazilian *Aldama* species (Asteraceae family) that were chosen because they are difficult to identify and have biological and pharmacological potential. All four species analysed could be differentiated on the basis of the set of anatomical features described for each species. So, we concluded that anatomy is able to provide data to assist with the taxonomic problems within the four species analysed herein.

Additional key-words: aerial organs, anatomy, secretory ducts, underground system

Introduction

Anatomical characteristics have proved to be an invaluable asset for supporting taxonomic studies on different families, providing information for establishing relationships and identifying plant taxa (Metcalfe and Chalk 1950;Scatena *et al*. 2005; de las Mercedes Sosa *et al*. 2014; Thadeo *et al*. 2014). For the Asteraceae family, anatomical features have been shown to have

considerable taxonomic value (Adedeji and Jewoola 2008; Duarte *et al*. 2008; Cury and Appezzato-da-Glória 2009; Inceer and Ozcan 2011; de las Mercedes Sosa *et al*. 2014), because the family includes plants that have various life forms and, thus, diverse anatomy (Karanović *et al*. 2015). Some studies have pointed out that anatomical features can also help clarify taxonomical problems in the *Aldama* La Llave genus, especially among Brazilian representatives (Bombo *et al*. 2012, 2014; Oliveira *et al*. 2013; da Silva *et al*. 2014).

The Heliantheae tribe and the Helianthinae subtribe, which includes the *Aldama* genus (=*Viguiera* Kunth), are well characterised by their reproductive morphological features (Robinson and Moore 2004; Magenta 2006). Generic delimitations are often not quite defined and as early as 1996, Schilling and Panero (1996) highlighted the ineffectiveness of studies based only on external morphological characteristics for solving taxonomic problems. *Viguiera sensu lato* is a proven polyphyletic group (Schilling and Panero 1991, 2011; Schilling *et al*. 2000; Robinson and Moore 2004; Magenta 2006) and, on the basis of molecular analysis, South American species of *Viguiera* were transferred to *Aldama* (Schilling and Panero 2011), because it is very evident that the Brazilian species form a cohesive group along with other South American representatives. However, problems in establishing general and specific boundaries still remain (Magenta *et al*. 2010).

Aldama genus, as established by Schilling and Panero (2011), includes 112 species that are distributed to south-western region of North America, from Mexico to South America. The Brazilian representatives comprise 35 species, of which 17 are endemic and occur mainly in savanna areas (cerrado; Magenta *et al*. 2010). The present study focuses on *Aldama bakeriana* S.F.Blake E.E.Schill. & Panero, *A. discolor* (Baker) E.E.Schill. & Panero, *A. grandiflora* (Gardner) E.E.Schill. & Panero and *A. squalida* (S.Moore) E.E.Schill. & Panero. The four species occur exclusively in cerrado physiognomies of central-western and south-eastern regions in Brazil (Magenta *et al*. 2010). These species were chosen because they are difficult to identify taxonomically if the specimens have no flowers (Magenta and Pirani 2014), they have biological and pharmacological potential (Marquina *et al*. 2001; Ambrosio *et al*. 2004; Arakawa *et al*. 2008; Nicolete *et al*. 2009; Porto *et al*. 2009; Carvalho *et al*. 2011) and they are representative species from key morphological groups in the *Aldama* genus and could help in future taxonomical investigations.

According to Magenta and Pirani (2014), *A. bakeriana* and *A. discolor* differ purely in terms of the features of the external involucral bracts of the capitula and cypsela pappus. *A. discolor* is also similar to *A. squalida* in regard to both habit and cypsela morphology. *A. grandiflora* was included in this analysis because its individuals are also very similar to those of *A. squalida*, at the vegetative stage, such that it is sometimes impossible to distinguish them (Magenta and Pirani 2014). Thus, the species cannot be distinguished purely on the basis of their reproductive features, and anatomical studies could help elucidate these delimitation problems.

Several studies have attempted to solve the taxonomical problems posed by the *Aldama* genus, generally on the basis of molecular and external morphological data. Because anatomical and

micro-morphological data are scarce, the aim of the present study was to contribute to the knowledge of the group and help solve the taxonomical problems faced. Aerial and underground vegetative organs from *A. bakeriana*, *A. discolor*, *A. grandiflora* and *A. squalida* are described herein for the first time and a comparative analysis is performed to highlight the unique features of each species and determine whether they can be distinguished in terms of anatomy.

Material and methods

Plant material

Samples from different populations of the four species chosen for the study were collected in 2012. *Aldama grandiflora* was collected in Brasília and Alto Paraíso de Goiás, State of Goiás (ESA123037, ESA123038, ESA123040), *A. bakeriana* in Alpinópolis, Divinópolis and Oliveira, State of Minas Gerais (ESA123041, ESA123042, ESA123043), *A. discolor* in Pedregulho, Sacramento and Uberlândia, State of Minas Gerais (ESA123044, ESA123045, ESA123046) and *A. squalida* in Campos Grande and Ribas do Rio Parto, State of Mato Grosso do Sul (SPF215969, SPF215970). The species were identified by a Brazilian specialist and the herbarium material was registered and incorporated into the collection at the Luiz de Queiroz School of Agriculture, University of São Paulo (ESA herbarium) and the University of São Paulo herbarium (SPF).

Anatomical analysis

Samples from leaves, aerial and underground stems, and roots were selected from each species and at least three different individuals were examined (one individual from each sampled population). The apex and the middle region of mature leaves were examined. Aerial-stem internodes of different diameters (the minimum – first internode, medium and maximum diameters) for each individual were examined. For underground organs, samples of different sizes were examined so as to understand organ development; roots of different diameters were analysed (tuberised and non-tuberised portions), as well as lateral roots. The tuberised portion was of uniform diameter along the root in *A. bakeriana* and *A. grandiflora*, whereas in *A. discolor* and *A. squalida*, there was some variation in diameter along the same root. Therefore, for these two species, the smallest and the largest diameter were sectioned. In addition, roots at different levels of development from the same individual and different individuals were also investigated.

All these samples were fixed in a formalin–acetic acid–50% ethanol solution (FAA 50, 5 : 5 : 18, by volume; Johansen 1940) or Karnovsky solution (Karnovsky 1965), placed in a vacuum pump to remove air from the tissue, dehydrated in a graded ethanol series and stored in 70% ethanol. The fixed samples were dehydrated in a graded ethanol series and embedded in plastic resin (Leica Historesin®, Heraeus-Kulzer, Hanau, Germany). The blocks were cross- and longitudinally sectioned (5–8 µm thick) using a rotary microtome (Model RM 2245, Leica Microsystems Nussloch GmbH,

Nussloch, Germany). Sections were stained with 0.05% toluidine blue O in a citrate–phosphate buffer, pH 4.5 (Sakai 1973), and mounted in Entellan® synthetic resin (Merck, Darmstadt, Germany). Prior to embedding in historesin, thick stem and xylopodium samples were softened in 10% ethylenediamine (Carlquist 1982). Cross-sections were also obtained by freehand cuts with a razor blade or using a sliding microtome that produced sections of 30–40 µm in thickness. These sections were clarified in a commercial solution of 20% sodium hypochlorite (v/v) diluted to 2.5% (w/w), and subsequently rinsed in distilled water, stained with safranin and astra blue (Bukatsch 1972) and mounted in glycerinated gelatin.

The epidermis dissociation technique using 10% Jeffrey solution was applied before observing the leaf surface (Johansen 1940). Fragments were stained with safranin and astra blue (Bukatsch 1972) and mounted in glycerinated gelatin.

Some histochemical analyses were performed on sections of fixed material, with some being embedded in historesin and some not. The following reagents and stains were used: Sudan IV for lipophilic substances (Jensen 1962), periodic acid Schiff (PAS) reaction for total polysaccharides (McManus 1948), ruthenium red for pectin and mucilage (Gregory and Baas 1989), zinc-chloride iodide for starch grains (Strasburger 1913), ferric chloride for phenolic compounds (Johansen 1940) and thymol-sulfuric acid reagent for detection of inulin cristals (Johansen 1940).

Photomicrographs were taken with a video câmera (DFC310Fx Leica Microsystems (Schweiz) AG, Heerbrugg, Switzerland) coupled to the Leica DMLB microscope. LAS 4.0 software (Leica) was used for image analysis.

For scanning electron microscopy, stem and leaf samples fixed in Karnovsky solution (Karnovsky 1965) were dehydrated in a graded ethanol series and subjected to critical-point drying with CO_2 (Horridge and Tamm 1969), mounted on aluminum stubs and coated with gold (30–40 nm thick). Observations were based on photomicrographs obtained using a scanning electron microscope (LEO, 150 435 VP, Zeiss, Oberkochen, Germany), operating at 20 kV.

Results

Anatomy of aerial organs

The leaf blades of the species analysed are covered by three types of trichome (Fig. 1*a*), namely, non-glandular trichome (NGT; Figs 1*b, c*), linear glandular trichome (LGT; Figs 1*d, e*) and capitate glandular trichome (CGT; Fig. 1*f*). The NGT is formed by three cells, including a dilated basal cell, a cylindrical intermediate cell of variable size and a terminal cell with an acute apex. The walls of the basal and intermediate cells are ornamented with wart-like structures composed of pectin (Fig. 1*b*); these are more evident in *A. bakeriana*. The base of this trichome is surrounded by sets of epidermal cells that exhibit pectin thickening and the number of sets can be different in a given species between its leaf surfaces and the number of sets can also vary among species (Fig. 1*c*, Table 1). The

LGT is uniseriate, moniliform, with a spatulate, globoid or tapering terminal cell. The median cells accumulate phenolic compounds (Fig. 1*d*), whereas the apical cells contain mucilage. The CGT is capitate and biseriate, consisting of one pair of basal cells and a variable number of cell pairs forming the head. A lipophilic secretion accumulates in the subcuticular space (Fig. 1*f*). Non-glandular trichomes and LGTs occur on both leaf surfaces in all four species, are mainly associated with the leaf veins, and are less frequent in *A. squalida*. Capitate glandular trichomes occur only on the abaxial epidermis of *A. discolor* and *A. grandiflora*.

The leaf is amphistomatic, bearing anomocytic stomata and, in front view (Figs 1*g–i*), the epidermal cells walls can be straight (Fig. 1*g*; adaxial epidermis of *A. grandiflora* and *A. squalida* and both leaf surfaces of *A. discolor*), straight to slightly sinuous (Fig. 1*h*; adaxial epidermis of *A. bakeriana* and abaxial epidermis of *A. grandiflora*) or sinuous (Fig. 1*i*; abaxial epidermis of *A. bakeriana* and *A. squalida*).

The leaf blade has a thin cuticle and one-layer epidermis whose cells have pectin-thickened external periclinal walls, mainly in the upper epidermis (Figs 1*j*, *k*). The mesophyll is dorsiventral (Fig. 1*j*; *A. discolor* and *A. squalida*) or isolateral (Fig. 1*k*; *A. bakeriana* and *A. grandiflora*) and, in this case, tends to be homogeneous. Lateral bundles occur along the leaf blade and can be surrounded by parenchymatous or lignified cells. They exhibit bundle-sheath extensions facing the upper and/or lower epidermis, with cells usually lignified. Secretory ducts are associated with the sheath extensions (Fig. 1*j*, *k*, arrows) and, in the same species, they can occur in both sheath extensions or only in the adaxial sheath extension. *Aldama bakeriana* was the only species in which the ducts occurred only in the adaxial sheath extension. Hydathodes are visible on the leaf margin of all species (Fig. 1*l*).

The midrib (Fig. 1*m–o*) has a projection filled with collenchyma, which is less prominent in *Aldama squalida* (Fig. 1*n*), and two to three layers of collenchyma are visible immediately below the lower epidermis. The vascular system is organised in a larger central collateral bundle and two (Fig. 1*m*) to four smaller collateral lateral bundles, surrounded by a sheath of parenchymatous (Fig. 1*o*) or lignified cells (Fig. 1*m*, *n*). In *A. discolor* and *A. squalida*, in addition to the lignified sheath cells, the bundles can exhibit fibre caps associated with both xylem and phloem (Fig. 1*n*). Secretory ducts are visible in the phloem and in the fundamental parenchyma (Fig. 1*o*). The degree of lignification and the number of secretory ducts in the midrib vary according to the specimen and/or species analysed.

Aerial-stem anatomy is very similar in all the species analysed (Table 1). In the primary structure (Fig. 2*a*), the same types of leaf trichome can be observed. Capitate glandular trichomes occur in *A. grandiflora*, *A. discolor* and *A. squalida*, but they are rare in the latter two species. Non-glandular trichomes and LGTs are observed in the stems of all four species. The stem epidermis is a single layer, with pectin-thickened walls, and is covered with a thin cuticle (Fig. 2*b*). The cortical region (Fig. 2*a*, *b*) is formed by layers of collenchyma immediately below the epidermis and cortical

parenchyma with secretory ducts, which can have a uni- or biseriate epithelium. It is delimited internally by endodermis with Casparian strips, the cells of which contain starch grains (Fig. 2*c*).

In the vascular cylinder, the pericycle is formed by a variable number of cell layers, and in the portion opposite the primary phloem of the vascular bundles, groups of smaller cells with thin or already lignified cell walls can be seen (Fig. 2*a*). In the interfascicular portion, the pericycle can appear to be interrupted by primary phloem cells (Fig. 2*d*). In all four species, there are open-type collateral bundles of different sizes, and some of these bundles can be projected outward from the others. In the larger bundles, the proto- and metaxylem consist of more rows (Fig. 2*a*) than they do in the smaller ones. There are secretory ducts in the primary phloem (Fig. 2*e*), except in *A. squalida*. The parenchymatous pith is wide and secretory ducts are visible in the perimedullary parenchyma, with uni- or bisseriate epithelium and lumina of variable size (Fig. 2*a*).

In the secondary structure, all trichome types are rare, and the epidermis remains unchanged (Fig. 2*f*). In the cortex, the collenchyma appears partially or completely lignified and, exclusively in *A. grandiflora*, new secretory ducts are formed near the endodermis, which remains unaltered (Fig. 2*f*). In the vascular bundle, pericyclic fibres opposite the phloem tissue are already differentiated in all species (Fig. 2*g*). In a feature exclusive to *A. bakeriana*, the pericycle forms a ring of sclerified cells, which can be discontinuous depending on the specimen analysed (Fig. 2*g*). Cambial activity is not very pronounced because these species do not undergo any substantial increase in diameter (Fig. 2*g*). Few secretory ducts are formed in the secondary phloem of *A. squalida* (Fig. 2*h*), and the secondary xylem of all species displays intense lignification of the rays and many fibres (Fig. 2*h*, *i*). The pith is widened and this enlargement contributes to the discrete increase in stem thickness (Fig. 2*j*). The secretory ducts already present in the pith of the primary structure appear to be positioned inside the medullary parenchyma because of the division of the perimedullary cells, and new ducts are formed next to protoxylem elements (Fig. 2*j*). The central portion of the pith was subject to lignification in all four species (Fig. 2*i*).

Underground-system anatomy

The underground systems of the four species analysed are very similar, both morphologically and anatomically. They consist of a thickened, bud-bearing organ that occurs in upper soil layers (Fig. 3*a*, *b*), and is responsible for producing all the roots (Fig. 4*a*, *b*). This organ is formed by articulated swollen nodes or globose internodes, corresponding to the swollen base of the aerial branch, which develops from axillary buds (Fig. 3*c*–*e*) located in the proximal region (Fig. 3*d*, *e*) during favourable periods of development. This process increases the anatomical complexity of the organ because the stem base of the aerial branches self-grafts (Fig. 3*f*), producing a horizontal structure. It is possible to observe the remaining carbonised bases of some aerial branches emitted in previous periods of underground-organ development (Fig. 3*b*). This organ varies in size from one

individual to another in a given population, probably because of the difference in developmental age (compare Fig. 3*a* with Fig. 3*b*).

The underground organs of the *Aldama* species studied are of stem-like origin, which was confirmed by the centrifugal development of the primary xylem (Fig. 3*f*, inset). The protective tissue is formed by the epidermis in younger regions, or by stratified cork in older regions. The inner cortical-parenchyma cells show periclinal divisions that produce new layers of stratified cork as the outer layers of cortex are eliminated, and at later stages of development, the secondary phloem is located immediately below the protective tissue (Fig. 3*g*). In the secondary phloem, secretory ducts are visible, interspersed with vascular elements, fibres and parenchyma cells (Fig. 3*g*, *h*). In larger organs found in *A. grandiflora* and *A. squalida*, a well developed secondary phloem with several series of secretory ducts is observed (Fig. 3*g*). In *A. grandiflora*, these ducts appeared larger than in the other species and the lumen is formed by separation and subsequent lysis of the cells, a process that increases the size of the lumen(Fig. 3*h*). The secondary xylem is also well developed, occupying the largest portion of the organ, with abundant parenchyma cells. Secretory ducts can also be observed in the medullary parenchyma (Fig. 3*i*). Fructans were visible in the vascular parenchyma of some samples, always associated with the vessel elements (Fig. 3*j*).

The root systems in all species analysed originate from the thickened organ described above and consist of adventitious roots with non-tuberised and tuberised portions, and lateral roots (Fig. 4*a*, *b*). The morphology of the roots vary from one species to another. In *A. bakeriana*, *A. discolor* and *A. squalida*, the non-tuberised portions are more slender than they are in *A. grandiflora* and the tuberised portions occur in the distal part of the roots, whereas in *A. grandiflora* they are located in the middle part (Fig. 4*a*, *b*). In *A. bakeriana*, *A. discolor* and *A. squalida*, the tuberised portion is short, ranging from cylindrical to globose in shape (Fig. 4*a*). The tuberous roots of *A. grandiflora* are more developed, elongated and fusiform (Fig. 4*b*).

The primary structure of the non-tuberised portions of the adventitious roots and lateral roots (Fig. 4*c*, *d*) consists of a one-layer epidermis, which is gradually eliminated, a cortex with a single-layer exodermis accumulating phenolic compounds and with parietal suberin thickening. The cortical parenchyma formed by five to seven layers of cells and an endodermis with Casparian strips (Fig. 4*c*). Thinner roots can exhibit mycorrhizal fungi (Fig. 4*d*). The ducts secreting lipophilic substances are located in the innermost cortical region and in areas opposite the primary phloem. Their epithelium is formed by two cells from the cortical parenchyma and two endodermal cells (Fig. 4*c*). In the vascular cylinder, the pericycle is uniseriate and the vessel elements are interspersed with fibres (Fig. 4*d*). Adventitious roots contain parenchymatous pith.

Although the anatomy of the non-tuberised portion (Fig. 4*e*–*g*) and lateral roots is similar, especially in regard to the protective tissue and cortical region, some differences are visible. The epidermis and exodermis are replaced by layers of suberised cells that originate from periclinal divisions of the cells in the layer beneath the exodermis. In larger roots, cortical parenchyma cells

undergo anticlinal divisions, the sclereids are differentiated and the lumina of the secretory ducts increase in size (Fig. 4*e*, *g*). The size of the secretory-duct lumen can vary from one species to another, and is wider in the larger-diameter roots of *A. discolor* and *A. squalida.* Cambial activity in the vascular cylinder is discrete and, initially, the formation of secondary vascular cells occurs only in the opposite region of the primary phloem and xylem (Fig. 4*g*). As it develops, the root becomes more lignified and the secondary vascular tissues form a continuous cylinder. The pith is parenchymatous in all four species, and more developed in the larger portions of the roots in *A. discolor* and *A. squalida* (Fig. 4*g*).

In the tuberised portions (Fig. 4*h*–*m*), the stratified cork formed by cells with suberised walls covers the roots (Fig. 4*h*), but only in *A. bakeriana* are new secretory spaces formed immediately below the protective tissue (Fig. 4*i*). The cortical organisation is similar to that described above. As the root tuberises, the vascular cylinder expands as the medullary parenchyma cells divide (Fig. 4*j*). There is little pericycle and cambial activity (Fig. 4*k*); pericycle cells increase in volume and divide, whereas the cambium is responsible for the formation of vascular parenchyma cells (mainly in more tuberised roots) and the formation of a few vascular elements (Fig. 4*l*). Secretory ducts are observed in the secondary phloem (Fig. 4*l*) of *A. discolor*, *A. grandiflora* and *A. squalida*, and secretory cavities, which appeared shorter and more isodiametric than the ducts, also form in the pith parenchyma of all species. Fructans accumulate throughout the tuberised structure, mainly in the parenchyma adjacent to the vascular tissues (Fig. 4*m*).

Discussion

All four species analysed were anatomically very similar. However, they could be distinguished on the basis of the set of anatomical features described for each species. The leaf characters that differed from one species to another included the outline of the anticlinal cell walls, the presence or absence of the glandular trichome, the series number of epidermal cells surrounding the base of the non-glandular trichome, the type of mesophyll and the occurrence and number of secretory ducts in the midrib and lateral bundle-sheath extension. For the aerial stem (the vegetative organ that was more anatomically similar in all species), the distribution of secretory structures was the distinctive character. In terms of underground organs, the morphology of the root tuberised region, and the occurrence and distribution of the secretory spaces were also helpful in distinguishing the species. The thickened underground stem was very similar in structure in all species, and was the only organ that did not contribute to distinguishing them.

Among the types of secretory structures found in representatives of the Asteraceae family (Castro *et al*. 1997; Fahn 2000), the occurrence of ducts, cavities, trichomes and hydathodes has been reported for the *Aldama* genus (Bombo *et al*. 2012; Oliveira *et al*. 2013; da Silva *et al*. 2014), and was confirmed for the species analysed in the present study. Because of morphological and anatomical

variations in these structures and on the basis of their highly consistent occurrence and distribution in plant organs, secretory structures are often pointed out as features of diagnostic value among species and genera (Solereder 1908; Metcalfe and Chalk 1950; Kelsey 1984; Castro *et al*. 1997; Fahn 2000). In our study, they were the main distinctive characteristics found.

The presence of particular types of trichome can help in the delimitation of species, genera and even families (Metcalfe and Chalk 1950). The occurrence of the NGT with cell walls ornamented with wart-like structures, and the LGT, have been confirmed as characteristics for the genus *Aldama* (Castro *et al*. 1997; Bombo *et al*. 2012; Aschenbrenner *et al*. 2013; Oliveira *et al*. 2013) and also for Heliantheae tribe (Aschenbrenner *et al*. 2013). However, the nature of the LGT secretion seems to vary from one species to another, both for the species analysed in the present study and also for other Brazilian *Aldama* species already investigated (Oliveira *et al*. 2013; da Silva *et al*. 2014).This trichome accumulates phenolic compounds and mucilaginous substances, whereas Aschenbrenner *et al*. (2013) and Spring *et al*. (1992) reported that terpenes were accumulated in the LGT in *Helianthus annuus*, which is a species closely related to *Aldama*.

Ducts, cavities and trichomes are structures widely reported for the Asteraceae family (Metcalfe and Chalk 1950; Ramayya 1962; Cury and Appezzato-da-Glória 2009) and recorded as a character common to this family, and also in the *Aldama* genus (Bombo *et al*. 2012; Oliveira *et al*. 2013). Ducts were found in all the vegetative organs of the species investigated herein, but cavities were found only in the roots, as post-formed structures produced by tuberisation. This pattern has also been observed in the other Brazilian *Aldama* species (Oliveira *et al*. 2013; Bombo *et al*. 2014; da Silva *et al*. 2014). In terms of the genus itself, the occurrence and distribution of secretory structures have been considered a relevant character for solving species identification problems within specific subgroups (da Silva *et al*. 2014) and among the species analysed herein.

The underground systems were very similar to those of other *Aldama* species already studied, both morphologically and anatomically (Oliveira *et al*. 2013; Bombo *et al*. 2014; da Silva *et al*. 2014). For these *Aldama* species, the thickened, bud-bearing underground systems were described as xylopodia. According to Rizzini (1965), a xylopodium is a perennial thickened woody organ with numerous buds and a high resprout capacity, formed by the tuberisation of the hypocotyl or the root–stem transition region and the proximal portion of the main root. Another feature of this organ is the production of stems that periodically lead to the formation of a self-grafting structure at the bases of the stem axes (Paviani 1987). Anatomically, they tend to be mostly woody tissues with no storage tissue, apart from the vascular parenchyma (Appezzato-da-Glória 2015). For the *Aldama* species studied herein, all the anatomical features indicated that the underground organ can be classified as a xylopodium. However, we did not investigate the origin of these organs, and, therefore, cannot confirm whether they originate from the hypocotyl or root–stem transition region. Our results indicated that the entire structure is formed by the swelling of the base of aerial branches developed from axillary buds, resulting in an organ similar to a xylopodium in structure, but not in origin.

The *Aldama* species chosen for the present study occur mainly in cerrado areas in Brazil (Magenta and Pirani 2014). The predominance of Asteraceae species with bud-bearing thickened underground systems is a striking feature in the cerrado biome (Warming 1908; Rizzini and Heringer 1961; Fidelis and Pivello 2011) and allows these plants to resprout quickly after damage by fire (Coutinho 1990; Fidelis and Pivello 2011). Although these organs are not typical reserve structures because they have no parenchymatous storage tissue, in the species studied herein, as well as in other *Aldama* species (Oliveira *et al*. 2013; Bombo *et al*. 2014; da Silva *et al*. 2014), the thickened underground organ is associated with tuberous roots, which in turn accumulate reserve as fructans. For these species, fire events seem to be very important in the development of the underground stem, because they stimulate the sprouting of axillary buds found in the swollen base of remaining carbonised branches. In *Chresta sphaerocephala* DC., the authors provided further evidence of how fire plays a part in the sprouting process, and, consequently, in the development of the underground organ. These species are said to have a diffuse underground system of radicular structure (Appezzato-da-Glória *et al*. 2008).

In regard to the tuberised roots, although morphological variations were found from one species to another, mainly in terms of the shape and size of the tuber (Oliveira *et al*. 2013; Bombo *et al*. 2014; da Silva *et al*. 2014), the anatomical findings indicated that the tuberisation process is similar in all species. In *Aldama*, tuberisation is due to the intense proliferation and increase in the volume of the medullary cells and, in some species such as *A. tenuifolia* (da Silva *et al*. 2014) and in the four species analysed herein, in addition to cell proliferation, the cambium also contributes significantly to the formation of the vascular parenchyma. This process differs from that described for other Asteraceae genera, such as *Vernonia oxylepis* Sch. Bip. in Mart. ex Baker, in which root tuberisation results from pericycle proliferation (Vilhalva and Appezzato-da-Glória 2006; Hayashi and Appezzato-da-Glória 2007), and *Smallanthus sonchifolius* (Poepp. & Endl.) H.Robinson, where it involves the division of cortical cells (Machado *et al*. 2004).

Over and above root tuberisation, the type of secondary protective tissue and the accumulation of fructans, both being reported in the underground organs, have also been confirmed as characters typical of the genus. Both the presence of stratified cork and fructan accumulation as a reserve source of carbohydrate are strictly related to controlling water loss and adaptation to the adverse conditions of the cerrado region. According to De Micco and Aronne (2012), the presence of suberised layers of cells covering the roots is an important mechanism for preventing the flow of water from the roots to the soil under extreme drought conditions. Fructans play an important role in osmotic regulation, especially in environments with temporary water deficit (Brocklebank and Hendry 1989; Figueiredo-Ribeiro 1993; Valluru and Van den Ende 2008; Vilhalva *et al*. 2011), because the ability to produce a rapid metabolic response and use these carbohydrates when environmental conditions become favourable is a distinct advantage to plants that accumulate these polysaccharides (Figueiredo-Ribeiro 1993; Vilhalva *et al*. 2011).

On the basis of the anatomical features exhibited, we can conclude that anatomical studies are able to provide data to help solve the taxonomic problems raised by the four species analysed herein, and also to corroborate the results of other studies on the *Aldama* genus (Bombo *et al*. 2012, 2014; Oliveira *et al*. 2013; da Silva *et al*. 2014). Although grouping the characteristics of different organs may be more efficient for species identification, we identified a set of foliar characters that, in combination with other morphological data, can be sufficient to correctly identify the four species analysed herein, obviating the need to remove the underground organs that are so important to the conservation of these species in the environment.

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References

Adedeji O, Jewoola O (2008) Importance of Leaf Epidermal Characters in the Asteraceae Family. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* **36**, 7-16.

Appezzato-da-Glória B, Cury G, Soares M, Rocha R, Hayashi A (2008) Underground systems of Asteraceae species from the Brazilian Cerrado. *Journal of the Torrey Botanical Society* **135**, 103-113.

Appezzato-da-Glória B (2015) 'Morfologia de sistemas subterrâneos de plantas. Morphology of plant underground systems.' (3i Editora: Belo Horizonte).

Aschenbrenner AK, Horakh S, Spring O (2013) Linear glandular trichomes of *Helianthus* (Asteraceae): morphology, localization, metabolite activity and occurrence. *AoB Plants* **5**, **:**plt028.

Blake SF (1918) A revision of the genus *Viguiera*. *Contributions from the Gray Herbarium of Harvard University* **51**, 1-205.

Bombo AB, Oliveira TS, Oliveira ADSS, Rehder VLG, Magenta MAG, Appezzato-da-Glória B (2012) Anatomy and essential oils from aerial organs in three species of *Aldama* (Asteraceae-Heliantheae) that have a difficult delimitation. *Australian Journal of Botany* **60**, 632-642.

Bombo AB, Oliveira TS, Oliveira ASS, Rehder VLG, Appezzato-da-Glória B (2014) Anatomy and essential oil composition of the underground systems of three species of *Aldama* La Llave (Asteraceae). *The Journal of the Torrey Botanical Society* **141**, 115-125.

Brocklebank KJ, Hendry GAE (1989) Characteristics of plant species which store different types of reserve carbohydrates. *New phytologist* **112**, 255-260.

Bukatsch F (1972) Bermerkungen zur Doppelfarbung Astrablau-Safranin. *Mikrokosmos* **61**, 255.

Carlquist S (1982) The use of ethylenediamine in softening hard plant structures for paraffin sectioning. *Stain Technology* **57**, 311-317.

Castro MDM, Leitão-Filho HDF, Monteiro WR (1997) Utilização de estruturassecretorasna identificação dos gêneros de Asteraceae de uma vegetação de cerrado. *Revista Brasileira de Botânica* **20**, 163-174.

Coutinho L (1990) Fire in the ecology of the Brazilian Cerrado. In 'Fire in the tropical biota – ecosystem process and global challenges. (Ed. J Goldamer) pp. 82-105. (Springer-Verlag: Berlin).

Cury G, Appezzato-da-Glória B (2009) Internal secretory spaces in thickened underground systems of Asteraceae species. *Australian Journal of Botany* **57**, 229-239.

De Micco V, Aronne G (2012) Morpho-anatomical traits for plant adaptation to drought: from morphological to molecular features. In 'Plant responses to drought stress. (Ed. R Aroca) pp. 37-61. (Springer: Berlin, Heidelberg)

Duarte M, Wolf S, de Paula B (2008) *Smallanthus sonchifolius* (Poepp.) H. Rob. (Yacon): microscopical identification of the leaf and stem for the pharmacognostic quality control. *Revista Brasileira de Ciências Farmacêuticas* **44**, 157-164.

Fahn A (2000) Structure and function of secretory cells. *Advances in Botanical Research Incorporating Advances in Plant Pathology* **31**, 37-75.

Fidelis A, Pivello V (2011) Deve-se Usar o Fogo como Instrumento de Manejo no Cerrado e Campos Sulinos? . *Biodiversidade Brasileira* **2**, 12-25.

Figueiredo-Ribeiro RCL (1993) Distribulção, aspectos estruturais e funcionais dos frutanos, com ênfase em plantas herbáceas do Cerrado. *Revista Brasileira de Fisiologia Vegetal* **5**, 203-208.

Gregory M, Baas P (1989) A survey of mucilage cells in vegetative organs of the dicotyledons. *Israel Journal of Botany* **38**, 125-174.

Hayashi A, Appezzato-da-Glória B (2007) Anatomy of the underground system in *Vernonia grandiflora* Less. and *V. brevifolia* Less. (Asteraceae). *Brazilian Archives of Biology and Technology* **50**, 979-988.

Horridge G, Tamm S (1969) Critical point drying for scanning electron microscopic study of ciliary motion. *Science* **163**, 817-818.

Inceer H, Ozcan M (2011) Leaf anatomy as an additional taxonomy tool for 18 taxa of *Matricaria* L. and *Tripleurospermum* Sch Bip. (Anthemideae-Asteraceae) in Turkey. *Plant Systematics and Evolution* **296**, 205-215.

Jensen W (1962) 'Botanical histochemistry, principles and practice.' (W. H. Freeman: San Francisco)

Johansen D (1940) 'Plant microtechnique.' (McGraw-Hill Book Co. Inc.: New York)

Karanovic D, Lukovic J, Zoric L, Anackov G, Boza P (2015) Taxonomic status of *Aster*, *Galatella* and *Tripolium* (Asteraceae) in view of anatomical and micro-morphological evidence. *Nordic Journal of Botany* **33**, 484-497.

Karnovsky MJ (1965) A Formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *The Journal of cell Biology* **27**, 137-8A.

Kelsey R (1984) Glandular trichomes - a helpful taxonomic character for *Artemisia nova* (Black sagebrush). *Journal of Range Management* **37**, 370-372.

Machado Sr, Oliveira Dmt, Dip Mr, Menezes Nl (2004) Morfoanatomia do sistema subterrâneo de *Smallanthus sonchifolius* (Poepp. & Endl.) H. Robinson (Asteraceae). *Revista Brasileira de Botânica* **27**, 115-123.

Magenta MAG (2006) *Viguiera* Kunth (Asteraceae, Heliantheae) na América do Sul e sistemática das espécies do Brasil. PhD thesis. Universidade de São Paulo, São Paulo.

Magenta MAG, Pirani JR (2014) Novidades taxonômicas em *Aldama* (Asteraceae-Heliantheae). *Rodriguésia* **65**, 175-192.

Magenta MAG, Pirani JR, Mondin CA (2010) Novos táxons e combinações em *Viguiera* (Asteraceae - Heliantheae). *Rodriguésia* **61**, 1-11.

Metcalfe CR, Chalk L (1950) 'Anatomy of the dicotyledons.' (Clarendon Press: Oxford)

Oliveira TSd, Bombo AB, Appezzato-da-Glória B (2013) Anatomy of vegetative organs with an emphasis on the secretory structures of two species of *Aldama* (Asteraceae – Heliantheae). *Botany* **91**, 335-342.

Paviani T (1987) Anatomia do desenvolvimento do xilopódio de *Brasilia sickii* G.M. Barroso. Estágio inicial. *Ciência e Cultura* **39**, 399-405.

Ramayya N (1962) Studies on the trichomes of some Compositae II. Phylogeny and Classification. *Bulletin of the Botanical Survey of India* **4**, 189-192.

Rizzini C (1965) Estudos experimentaissobre o xilopódio e outros órgãos tuberosos em plantas do Cerrado. *Anais da Academia Brasileira de Ciências* **37**, 87-113.

Rizzini C, Heringer E (1961) Underground organs of plants from some southern Brazilian savannas, with special reference to the xylopodium. *Phyton* **17**, 105-124.

Robinson H, Moore A (2004) New species and new combinations in *Rhysolepis* (Heliantheae: Asteraceae). *Proceedings of the Biological Society of Washington* **117**, 423-446.

Sakai W (1973) Simple method for differential staining of paraffin embedded plant material using Toluidine Blue O. *Stain Technology* **48**, 247-249.

Scatena V, Giulietti A, Borba E, Van den Berg C (2005) Anatomy of Brazilian Eriocaulaceae: correlation with taxonomy and habitat using multivariate analyses. *Plant Systematics and Evolution* **253**, 1-22.

Schilling E, Panero J (1991) Evidence for a close relationship between *Iostephane* and *Viguiera* (ASTERACEAE, HELIANTHEAE). *American Journal of Botany* **78**, 1054-1062.

Schilling E, Panero J (1996) Phylogenetic reticulation in subtribe Helianthinae. *American Journal of Botany* **83**, 939-948.

Schilling E, Panero J (2011) A revised classification of subtribe Helianthinae (Asteraceae: Heliantheae) II. Derived lineages. *Botanical Journal of the Linnean Society* **167**, 311-331.

Schilling EE, Da Costa FB, Lopes NP, Heise PJ (2000) Brazilian species of *Viguiera* (Asteraceae) exhibit low levels of its sequence variation. *Edinburgh Journal of Botany* **57**, 323-332.

Silva EMS, Hayashi AH, Appezzato-da-Glória B (2014) Anatomy of vegetative organs in *Aldama tenuifolia*and *A. kunthiana* (Asteraceae: Heliantheae). *Brazilian Journal of Botany* **37**, 505-517.

Solereder H (1908) 'Systematic anatomy of the dicotyledons: A handbook for laboratories of pure and applied botany.' (Clarendon Press: Oxford)

Sosa MdlM, Pico GMVd, Dematteis M (2013) Comparative anatomy of leaves and stems in some species of the South American genus *Chrysolaena* (Vernonieae, Asteraceae) and taxonomic implications *Nordic Journal of Botany* **32**, 611-619.

Spring O, Rodon U, Macias F (1992) Sesquiterpenes from noncapitate glandular trichomes of *Helianthus annuus. Phytochemistry* **31**, 1541-1544.

Strasburger E (1913) 'Handbook of practical botany.' 7th ed. (George Allen: London)

Thadeo M, Azevedo A, Meira R (2014) Foliar anatomy of neotropical Salicaceae: potentially useful characters for taxonomy. *Plant Systematics and Evolution* **300**, 2073-2089.

Valluru R, Van den Ende W (2008) Plant fructans in stress environments: emerging concepts and future prospects. *Journal of Experimental Botany* **59**, 2905-2916.

Vilhalva D, Cortelazzo A, Carvalho A, Figueiredo-Ribeiro R (2011) Histochemistry and ultrastructure of *Campuloclinium chlorolepis* (Asteraceae) tuberous roots accumulating fructan: evidences of functions other than reserve carbohydrate. *Australian Journal of Botany* **59**, 46-52.

Vilhalva DAA, Appezzato-da-Glória B (2006) Morfoanatomia da raiztuberosa de *Vernonia oxylepis* Sch. Bip. in Mart. ex Baker – Asteraceae. *Acta Botanica Brasilica* **20**, 591-598.

Warming E (1908) Lagoa Santa - Contribuição para a geographiaphytobiologica. In 'Lagoa Santa e a vegetação de cerradosbrasileiros. (Eds E Warming and M Ferri). (EDUSP: São Paulo).

Table 1. Anatomical characters of the leaves, aerial stems and roots of *Aldama bakeriana, A. discolor, A. grandiflora* and *A. squalida*

ab, abaxial epidermis; ad, adaxial epidermis; CG, capitate glandular trichome; LG, linear glandular trichome; NG, non-glandular trichome

Parameter	*A. bakeriana*	*A. discolor*	*A. grandiflora*	*A. squalida*
Leaf				
Trichome type	NG and LG	NG, LG and CG	NG, LG and CG	NG and LG
Set of epidermal cells surrounding the NGT base	1 to 2 – ad 0 to 2 – ab	0 to 1 – ad 0 to 1 – ab	1 to 2 – ad 1 to 2 – ab	1 – ad 1 – ab
Anticlinal cell walls of the epidermis (ad/ab)	Straight to slightly sinuous/sinuous	Straight/straight	Straight/straight to slightly sinuous	Straight/sinuous
Mesophyll type	Isolateral	Dorsiventral heterogeneous	Isolateral	Dorsiventral heterogeneous
Number of secretory ducts in the midrib	2	2	2 to 6	2 to 4
Secretory ducts associated with sheath extensions (face)	Adaxial	Both	Both	Both
Stem				
Secretory ducts in primary phloem	Present	Present	Present	Absent
Secretory ducts in secondary phloem	Absent	Absent	Absent	Present
Root				
Shape of the tuberised portion	Short and cylindrical to globose	Short and cylindrical to globose	Elongated and fusiform	Short and cylindrical to globose
Formation of new secretory spaces in the cortical region of the tuberised portion	Present	Absent	Absent	Absent
Secretory ducts in secondary phloem	Absent	Present	Present	Present

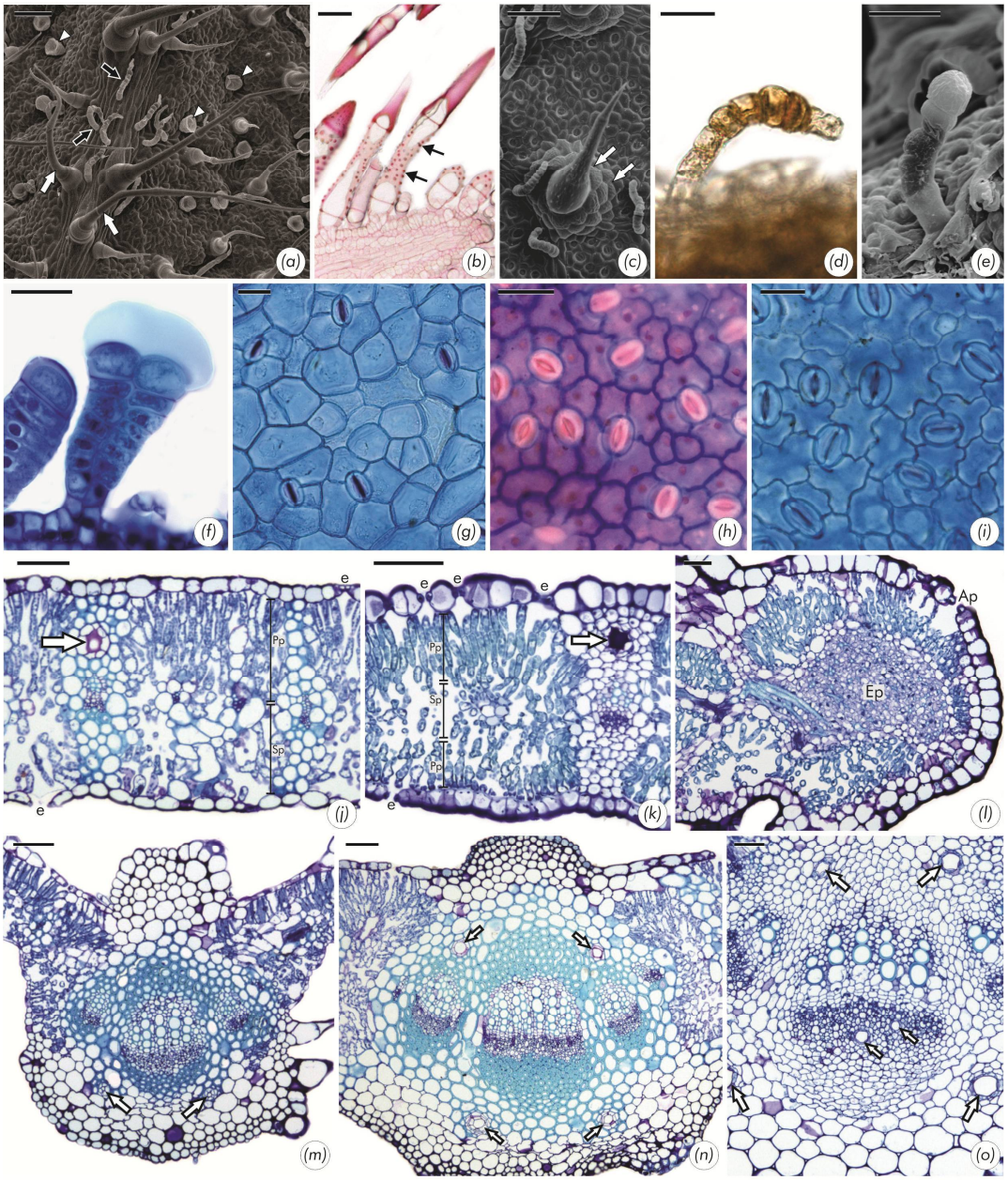

Fig. 1. **(***a, g, l*) *Aldama discolor*, (*b, d, m*) *A. bakeriana*, (*c, e, f, h, k, o*) *A. grandiflora* and (*i, j, n*) *A. squalida.* Scanning electron micrographs of (*a, c, e*) leaf surfaces and (*b, d, f–o*) light microscopy of leaves. (*a*) Abaxial surface covered by non-glandular (white arrows), linear glandular (black arrows) and capitate glandular (arrowheads) trichomes. (*b*) Non-glandular trichome (NGT), showing wart-like parietal ornamentation stained with ruthenium red (arrows). (*c*) Sets of epidermal cells, indicated by arrows, surrounding the NGT base. (*d, e*) Linear glandular trichome. (*f*) Capitate glandular trichome. (*g–i*) Front view of the epidermis, with (*g*) straight, (*h*) straight to slightly sinuous and (*i*) sinuous cell walls. (*j–o*) Cross-sections of the leaf blade. (*j*) Dorsiventral heterogeneous mesophyll. (*k*) Isolateral mesophyll. (*l*) Hydathode in the leaf margin. (*m–o*) Cross-sections of the midrib. Arrows in *j, k, m–o* indicate secretory ducts. e, stomata; Ap, aquiferous pore; Ep, epithem; Pp, palisade parenchyma; and Sp, spongy parenchyma. Scale bars = 100 µm (*a, c, j, k, m, n*), 50 µm (*b, l, o*) and 30 µm (*d–i*).

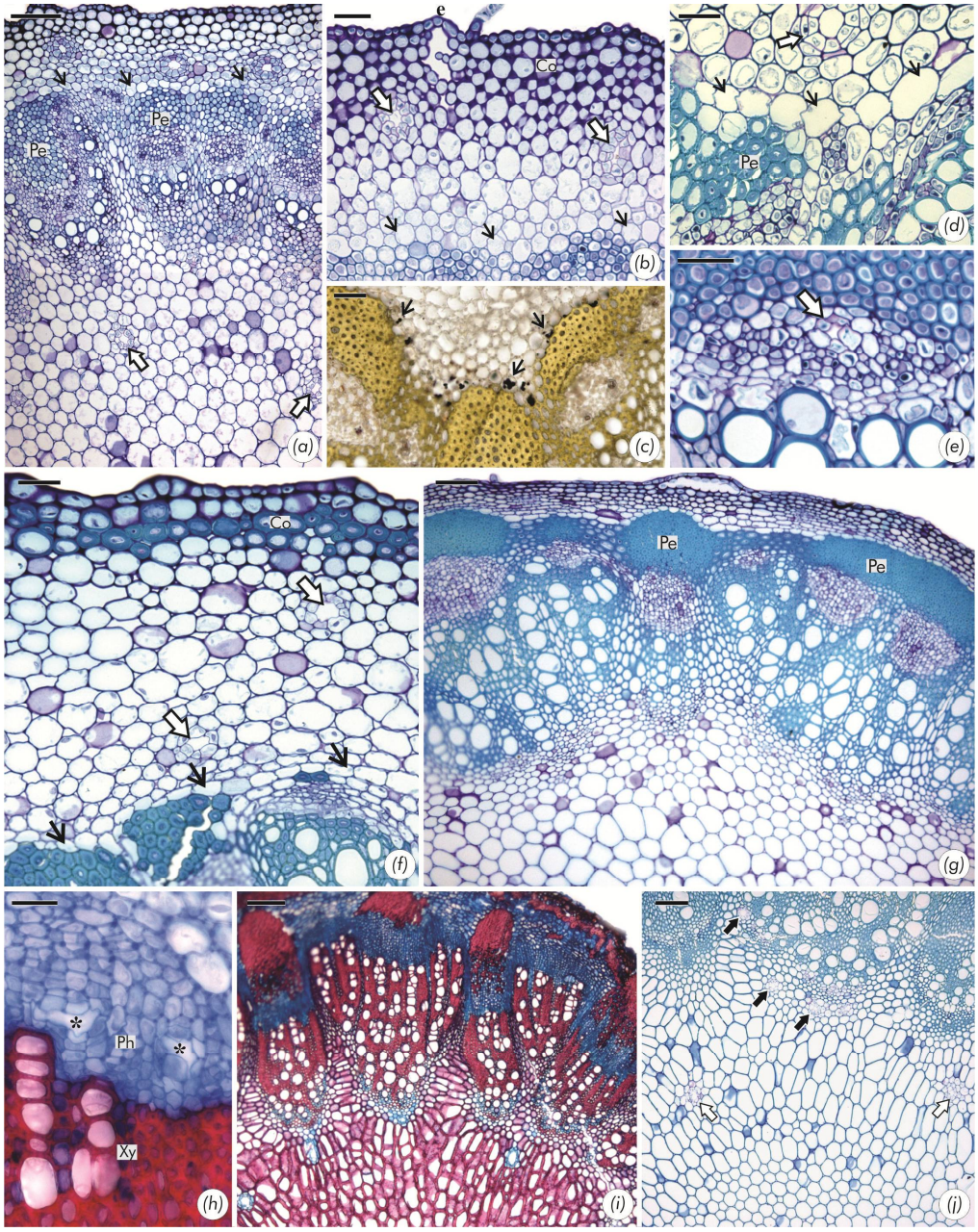

Fig. 2. Light microscopy cross-sections of the aerial stem of (*a, e*) *Aldama discolor*, (*b, c, f*) *A. grandiflora*, (*d, h–j*) *A. squalida* and (*g*) *A. bakeriana*. (*a–e*) Stem primary structure. (*a*) General view. (*b*) Detail of the epidermis and cortical region. (*c*) Starch grains in the endodermis cells, stained by iodine zinc chloride. (*d*) Primary phloem cells interrupting the pericycle. (*e*) Secretory ducts in the primary phloem. (*f–j*) Secondary structure of the aerial stem. (*f*) Epidermis and cortical region. (*g*) General view, showing low cambial activity. (*h*) Secretory duct (*) in the secondary phloem. (*i*) Secondary xylem with intense lignification of the rays, many fibres and lignified pith. (*j*) Divisions of the perimedullary cells and new ducts in the pith-adjoining protoxylem region (black arrows). White arrows in *a, b, d–f, j* indicate secretory ducts. Black arrows in *a–d, f* indicate the endodermis and in (*j*), new secretory ducts next to protoxylem elements. Co, collenchyma; Pe, pericyle; Ph, secondary phloem; and Xy, secondary xylem. Scale bars = 100 µm (*a, g, j*), 50 µm (*b, c, f*), 30 µm (*d, e, h*) and 200 µm (*i*).

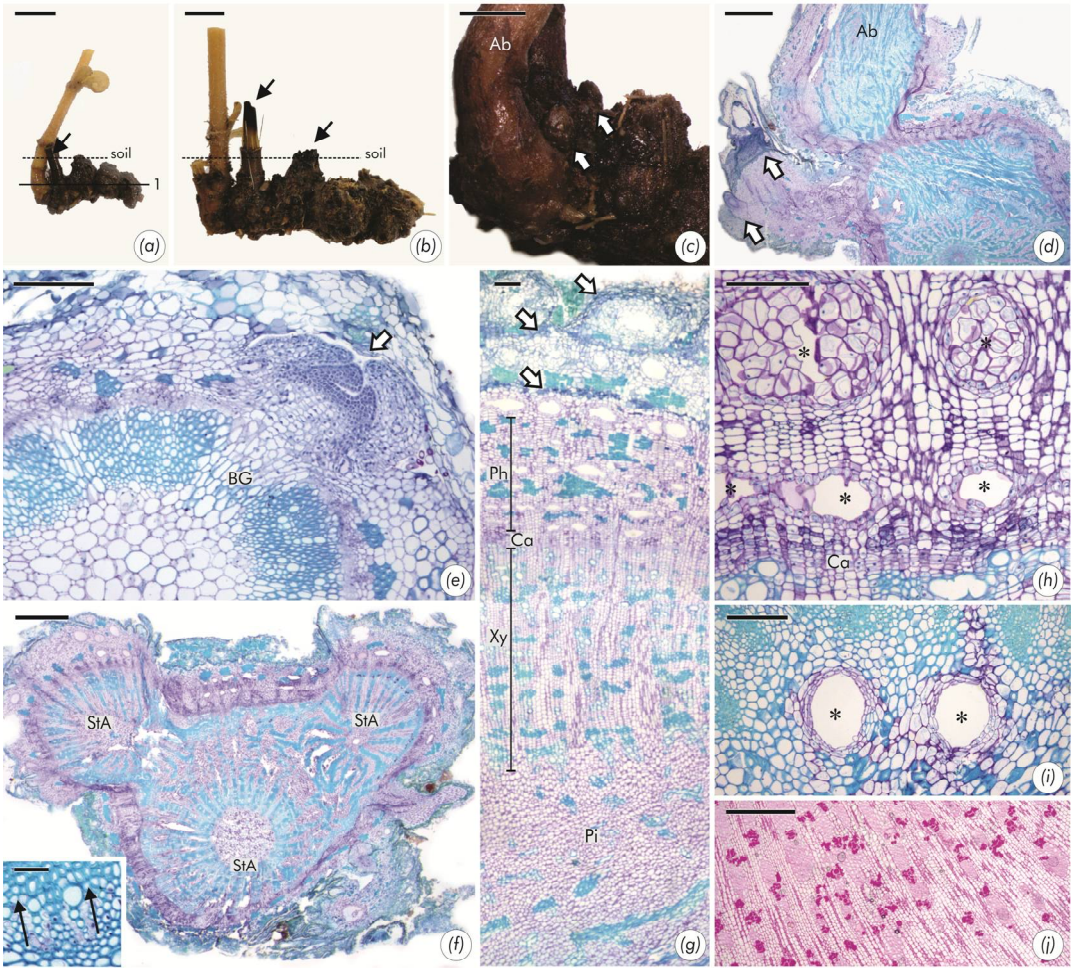

Fig. 3. (*a–c*) General view of the underground stem of *Aldama discolor* at (*a*, *b*) two different developmental stages. (*c*) Detail of the axillary buds (arrows). (*d–j*) Longitudinal sections (*d*) and cross-sections of the underground stem of (*f*) *A. discolor*, (*d* ,*h*, *i*) *A. grandiflora*, (*e*) *A.bakeriana* and (*f*, inset, *g*, *j*) *A. squalida*. (*d*) Axillary buds (arrows) located in the proximal region of aerial branches. (*e*) Axillary bud in cross-section, showing the gap (BG) in the vascular cylinder. (*f*) Self-grafting of stem axis (StA) and primary xylem exhibiting centrifugal maturation (inset). (*g*) General view showing stratified cork produced several times (arrows) and outer layers eliminated as the organ develops. (*h*) Secondary phloem with detail of the secretory ducts formed by separation and subsequent lysis of the cells. (*i*) Secretory structures in parenchymatous pith. (*j*) Fructans stained by periodic acid Schiff (PAS) reagent in the vascular parenchyma. Ab,= axillary bud; Ca, cambium; Ph, phloem; Pi, pith; and Xy, xylem. Asterisk in *h* and *i* indicates secretory spaces. Scale bars = 1 cm (*a*, *b*), 5 mm (*c*), 2 mm (*d*), 200 µm (*e*, *g–i*), 1 mm (*f*), 50 µm (inset) and 500 µm (*j*).

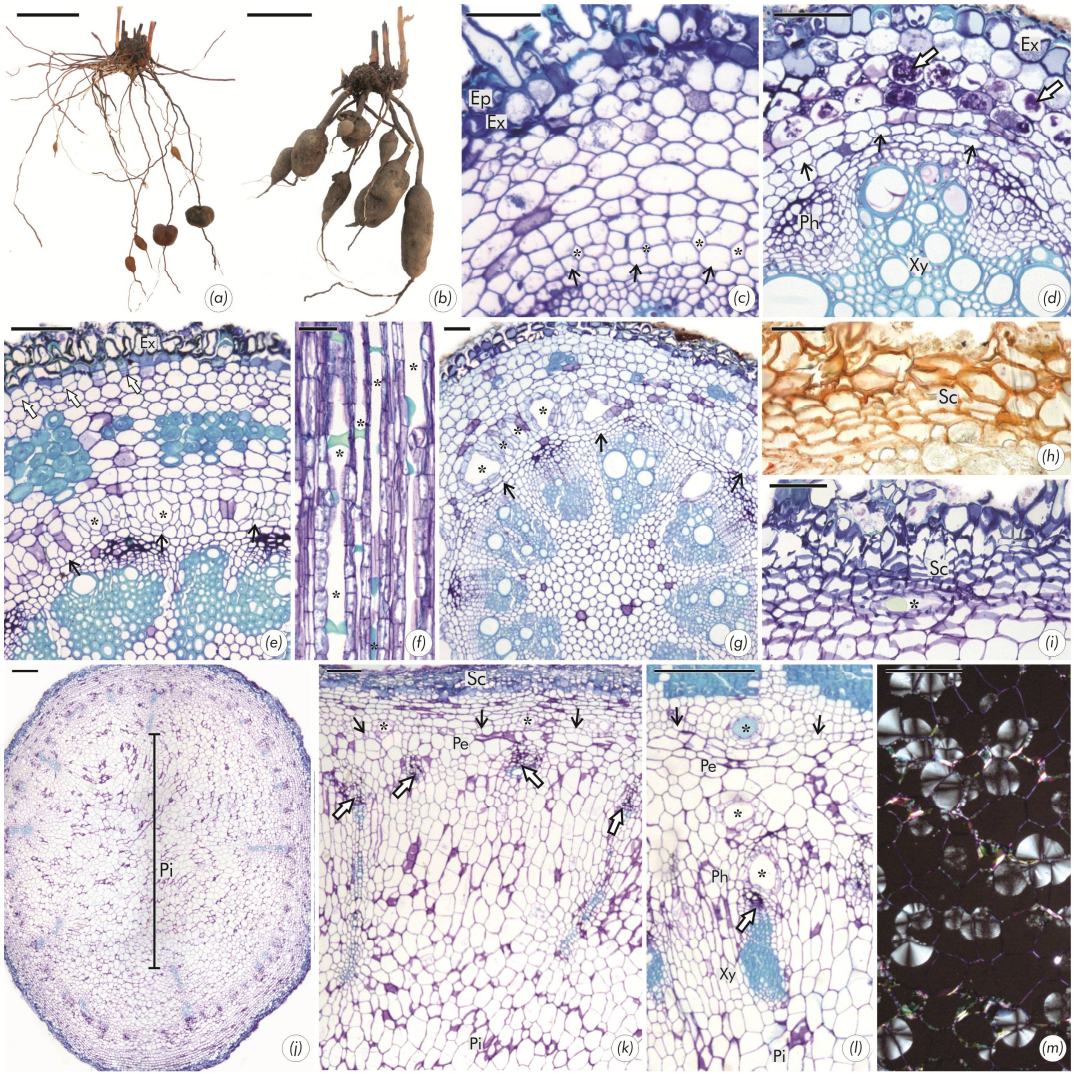

Fig. 4. (*a, j, k*) *Aldama discolor*. (*b–c, e, l*) *A. grandiflora.* (*d, f, g, m*) *A. squalida*. (*h, i*) *Aldama bakeriana*. (*a, b*) General view of the underground system of (*a*) *A. discolor* and (*b*) *A. grandiflora*, with tuberised adventitious roots. (*c–m*) Cross-sections and (*f*) longitudinal section of *Aldama* roots. (*c, d*) Lateral root. White arrows in *d* indicate the presence of fungi in the cortical parenchyma. (*e–g*) Non-tuberised portion of the root; secondary structure. White arrows in *e* show periclinal divisions of the cells in the layer beneath the exodermis. (*h–m*) Tuberised portion of the root. (*h*) Stratified cork stained by Sudan IV, indicating suberised cell walls. (*i*) New secretory space formed in the cortical region. (*j*) General view of the tuberised portion, with wide parenchymatous pith. (*k*) Cortical and vascular region. White arrows indicate discrete cambial activity. (*l*) Detail of the vascular cylinder with secretory ducts in the secondary phloem. (*m*) Inulin crystals under polarised light. Black arrows in *c–e, g, k, l* indicate the endodermis. Asterisk in *c–g, i, k, l* indicate internal secretory spaces. Ep, epidermis; Ex, exodermis; Pe, pericycle; Pi, pith; Ph, phloem; Sc, stratified cork; and Xy, xylem. Scale bars = 5 cm (*a, b*), 30 µm (*c, h*), 50 µm (*d–f, i*), 100 µm (*g, m*) and 200 µm (*j–l*).

CAPÍTULO II

Comparação morfoanatômica entre populações naturais de *Aldama grandiflora* (Asteraceae) de duas localidades no estado de Goiás

Comparação morfoanatômica entre populações naturais de *Aldama grandiflora* (Asteraceae) de duas localidades no estado de Goiás

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RESUMO

Fatores ambientais relacionados ao clima e ao solo, além do relevo e a altitude do local, exercem significante influência sobre a morfologia e anatomia das plantas. Os órgãos vegetativos aéreos são os que se encontram mais expostos às condições ambientais e podem refletir adaptações específicas para o local onde o indivíduo se localiza, uma vez que a plasticidade fenotípica está relacionada com a capacidade de um organismo em alterar a sua fisiologia ou morfologia devido a interações com fatores ambientais. Diversos estudos anatômicos têm sido publicados para espécies brasileiras do gênero *Aldama*, pertencente à família Asteraceae, os quais têm abordado apenas os aspectos qualitativos anatômicos nessas espécies e estudos que abordem as características quantitativas não são encontrados na literatura para o gênero. Entre as espécies brasileiras do gênero, *A. grandiflora* possui ampla ocorrência e distribuição geográfica, e por isso supõe-se que seus indivíduos estejam submetidos à condições ambientais e abióticas diferentes. Essa espécie foi escolhida para avaliarmos se as condições ambientais e edáficas podem alterar a morfometria entre indivíduos de populações e localidades diferentes. Para isso, seis populações provenientes de duas localidades de Goiás foram amostradas e parâmetros morfométricos foram avaliados para folhas e caules. Também houve amostragem de solo e coleta de dados climáticos para as seis populações. As análises indicaram que apesar de haver uma variação nas condições edafo-climáticas entre as duas localidades amostradas, a plasticidade fenotípica em resposta a essas condições foi pouco refletida em nível anatômico entre os indivíduos amostrados.

Palavras-chave: Cerrado, Compositae, morfometria, plasticidade fenotípica

INTRODUÇÃO

Representando cerca de 10% da flora mundial (Panero & Crozier 2012), a família Asteraceae é uma das mais importantes entre as angiospermas (Magenta 2006), compreendendo cerca de 1620 gêneros e 23600 espécies (Stevens 2001). Devido à grande dimensão da família, os nichos ocupados pelas suas espécies são bem variados, o que reflete em consideráveis diferenças anatômicas

que podem ser explicadas pela plasticidade fenotípica (Metcalfe & Chalk 1983), permitindo adaptações à diferentes ambientes (Cronquist 1981). A tribo Heliantheae, que ocorre no Cerrado brasileiro, é a segunda maior tribo da família, com 189 gêneros e cerca de 2500 espécies. Entre os seus representantes encontra-se *Aldama* La Llave, um gênero sul-americano, que conta com 35 espécies brasileiras, das quais 17 são endêmicas (Magenta et al. 2014), e cujos representantes se assemelham morfologicamente, causando confusão e problemas de delimitação taxonômica entre as espécies (Schilling & Panero 2011).

Aldama grandiflora (Gardner) E.E.Schill. & Panero (= *Viguiera grandiflora*) é um representante do gênero que possui ampla distribuição nas áreas abertas do Cerrado ocorrendo no Distrito Federal, norte do Mato Grosso do Sul, noroeste e nordeste de Goiás, noroeste de Minas Gerais e sul da Bahia (Magenta & Pirani 2010). Essa espécie tem destaque em *Aldama*, pois, além de sua ampla ocorrência, possui potencial resinífero (Magenta & Pirani 2010) e alto rendimento de óleos essenciais (Bombo et al. em preparação, capítulo 3), com diversos compostos com comprovada atividade biológica (Leite et al. 2007, Canales et al. 2008; Santoset al. 2011), o que confirma o potencial da espécie e do gênero. A espécie é caracterizada por variações estruturais intraespecíficas e alto grau de polimorfismo em relação à forma e cor da lâmina foliar, pois os seus representantes são tolerantes a diferentes intensidades luminosas (Magenta & Pirani 2014). Diversos estudos com finalidade taxonômica têm sido publicados com espécies brasileiras do gênero *Aldama* (Bombo et al. 2012, 2014; Oliveira et al. 2013; Silva et al. 2014), mas esses estudos têm abordado apenas os aspectos qualitativos anatômicos nessas espécies. Estudos que abordem as características quantitativas não são encontrados na literatura para o gênero.

Fatores ambientais relacionados ao clima, como relações hídricas, fotoperíodo e intensidade de luz, e também relacionados ao solo, como suas características físicas, químicas, além do relevo e a altitude do local, exercem significante influência sobre a morfologia e anatomia das plantas (Givnish 1984). De acordo com Scheiner (1993) e Stearns (1989), a plasticidade fenotípica está relacionada com a capacidade de um organismo em alterar a sua fisiologia ou morfologia devido a interações com fatores ambientais. Uma espécie como *A. grandiflora*, que possui plantas que apresentam alta capacidade fenotípica em caracteres que são relacionados à sobrevivência, têm grandes vantagens adaptativas quando expostas a ambientes que não apresentam as condições adequadas para o seu desenvolvimento (Gardoni et al. 2007). Sendo assim, essas alterações podem condicionar a planta a maiores níveis de tolerância ambiental, favorecendo a exploração das mesmas a novos nichos (Stearns 1989; Scheiner 1993).

Os órgãos vegetais que se encontram mais expostos às condições ambientais são as folhas, sendo suas mudanças estruturais, devido à plasticidade fenotípica, interpretadas como adaptações específicas para o local onde o indivíduo se localiza (Fahn 1986, Dickison 2000). Características caulinares, principalmente relacionadas ao sistema vascular, que são parâmetros que determinam a eficiência e resistência na condução de água (Kuniyoshi 1993), também podem

apresentar variações em função, por exemplo, da umidade relativa, temperatura e salinidade (Yaltirik 1970; Carlquist 1988; Bass 1982) e, portanto são fundamentais na manutenção desses indivíduos no ambiente.

Como *Aldama grandiflora* é bem representativa entre as espécies brasileiras do gênero, pela sua ampla ocorrência e distribuição geográfica, supõe-se que seus indivíduos estejam submetidos a condições ambientais e abióticas diferentes, e que suas características morfoanatômicas possam variar de acordo com a localidade em que se encontram. Dessa forma, esse estudo visou avaliar se: a) os parâmetros biométricos e morfométricos das folhas e dos caules de indivíduos de *A. grandiflora* provenientes de seis populações diferentes, amostradas em duas localidades distantes entre si, variam entre as populações, e b) se a variação que ocorre entre as populações, poderia ser reflexo da distribuição geográfica e, portanto, das condições edafo-climáticas as quais essas populações estão submetidas. As respostas dessas questões devem proporcionar um melhor entendimento das relações entre morfologia dos órgãos aéreos vegetativos e fatores ambientais para espécies de *Aldama* com ampla distribuição geográfica.

MATERIAL E MÉTODOS

Material botânico e área de coleta

Folhas e caules foram amostrados a partir plantas adultas provenientes de seis populações diferentes e distantes entre si, sendo que três populações foram coletadas na Localidade 1 (Brasília – DF e Planaltina-GO) e as outras três na Localidade 2 (Alto Paraíso de Goiás – GO) (Figura 1), totalizando 60 indivíduos. Para cada população, dez indivíduos foram amostrados. As duas localidades distam entre si mais de 200 km. As três populações amostradas para compor a Localidade 1 se encontravam em beiras de estradas, em resquícios da vegetação original, caracterizada por fisionomia de Cerrado (Figura 2A-C) e em todas elas, havia indício de passagem de fogo. Já as três populações que compuseram a Localidade 2 (Figura 2D-F) diferiram um pouco entre si, sendo que a população 4 foi amostrada em área urbana, também em beira de estrada, em terreno com indícios de passagem de maquinário agrícola; a população 5 foi amostrada em uma área de aceiro, dentro do Parque Nacional (PARNA) da Chapada dos Veadeiros e por fim, a população 6, foi amostrada em uma faixa de remanescente de Cerrado, entre uma estrada de chão e uma propriedade particular dentro do PARNA, e foi a população que apresentou menor número de indivíduos. Também havia indícios de passagem de fogo nessa localidade.

As informações detalhadas dos locais de coleta e coordenadas geográficas se encontram na Tabela 1. Vouchers foram depositados no Herbário da Escola Superior de Agricultura “Luiz de Queiroz” (ESA), da Universidade de São Paulo.

Análises anatômicas

Todo material proveniente do campo foi fixado em FAA 50 (formaldeído, ácido acético e álcool etílico 50%, na proporção 1:1:18 (Johansen 1940) e submetido à bomba de vácuo para retirada do ar contido nos tecidos. Para cada indivíduo coletado foi selecionada uma folha completamente expandida e de tamanho correspondente ao tamanho médio descrito para a espécie por Magenta (2006), e a partir dessa folha foram selecionadas amostras do terço médio, na região da nervura central e internervural. As amostras de caule foram coletadas na porção próximo ao solo, sendo que para cada população, três indivíduos foram amostrados. As amostras foram desidratadas em série etílica e infiltradas em historresina (Leica Historesin®) segundo as instruções do fabricante. Os blocos obtidos foram seccionados em micrótomo rotativo manual (Leica®) com navalha do tipo C. As seções foram coradas com azul de toluidina 0,05% (Sakai 1973) em tampão fosfato e citrato pH 4,5 e as lâminas montadas em resina sintética Entellan® (Merck®). Também foram utilizados o Sudan IV (Jensen 1962) para detecção de substâncias lipofílicas e o vermelho de rutênio, para substâncias pécticas e mucilaginosas (Johansen 1940).

Para a contagem de estômatos e de células epidérmicas ordinárias, em vista frontal da epiderme, visando o cálculo da densidade estomática, foi realizada a técnica de dissociação de epiderme em solução de Jeffrey (Johansen 1940). Os fragmentos foram corados com safranina e azul de astra (Bukatsch 1972) e montados em gelatina glicerinada. Essas lâminas foram utilizadas para a contagem de estômatos e de células epidérmicas ordinárias, em vista frontal da epiderme, para posterior cálculo do índice estomático (IE). O cálculo do IE foi feito de acordo com a fórmula: Índice Estomático (IE) = NE/(CE + NE), em que NE é o número de estômatos e CE o número de células epidérmicas (Cutter 1986). A medição da área foliar foi realizada com auxilio do equipamento Area Meter Modelo Li-3000, fabricante Li-Cor Inc..

Os seguintes parâmetros foram obtidos a partir das seções transversais do limbo foliar e dos entrenós: espessura da cutícula da face adaxial; espessura das células epidérmicas e da parede periclinal externa de ambas as faces; espessura do mesofilo; número de canais no parênquima homogênero da nervura central; altura da nervura central; diâmetro do entrenó; número de camadas e espessura do córtex; número de canais no córtex; diâmetro do cilindro vascular. Para cada parâmetro, foram realizadas cinco medidas/contagens, a fim de se obter uma média de cada indivíduo.

A captura das imagens foi realizada a partir das lâminas usando câmera de vídeo Leica DFC310Fx acoplada ao microscópio Leica® DM LB, com o auxilio do softawere LAS 4.0 software. Para as medições e contagens foi utilizado o Software Image J (Rasband 2006).

Amostragem e análise de solo

Para cada população foi realizada a amostragem de solo para análises físicas e químicas. Em cada local de coleta (Tabela 1) foram realizadas dez amostragens simples em uma profundidade de 0-20 cm, com auxílio de trado tipo sonda, modelo S-60 SONDATERRA®. As dez amostras simples

de cada profundidade foram misturadas e, por fim, foi retirada uma única amostra composta para cada população, contendo cerca de 500 g. Essas amostras foram devidamente embaladas e enviadas ao Laboratório de Análise de Solo, do Departamento de Ciência de Solo, da ESALQ/USP, para análise de sua composição química e física. As análises realizadas foram a SF2, que avalia a granulometria do solo, a SQ6, a fim de detectar e contabilizar os micronutrientes, e também a análise SQ7, para fins de classificação do solo.

Dados ambientais

Os parâmetros ambientais foram obtidos através do WorldClim (Site Worldclim). As variáveis: temperatura média anual, precipitação anual, e a altitude (elevação acima do nível do mar) foram coletadas. Os valores obtidos representam uma interpolação de dados observados entre 1950 e 2000.

Análises estatísticas

Todas as medidas obtidas foram submetidas a ANOVA e quando necessário os dados foram transformados por Log x. As médias obtidas para cada parâmetro, em cada população, foram submetidas ao teste de Tukey ($p < 0,05$). Também foi calculada a correlação entre as variáveis morfométricas e as edafo-climáticas. Todas as análises foram realizadas no software Statistica (Statsoft Inc. 2007).

RESULTADOS

Morfometria foliar e caulinar

Entre os parâmetros foliares avaliados, a espessura da cutícula da face adaxial do limbo foliar apresentou diferença estatística significativa entre as duas localidades e igualdade entre as populações de uma mesma localidade (Tabelas 2). Para os outros parâmetros, a variação observada não se restringiu às localidades de estudo, ou seja, populações de uma ou outra localidade apresentaram a mesma condição para um determinado parâmetro. Além da espessura da cutícula, os parâmetros que diferiram significativamente entre as populações foram o índice estomático de ambas as faces da folha, a espessura da nervura central, que apresentou correlação positiva com a espessura da parede periclinal da epiderme adaxial ($r = 0,8357$; $p= 0,38$), o número de canais no parênquima da nervura central e a espessura do mesofilo. A área foliar, que não apresentou diferenças entre as populações, apresentou correlação negativa com o índice estomático da face abaxial ($r = - 0,8699$; $p = 0,24$).

Os parâmetros caulinares foram mais similares entre si (Tabela 3), sendo que não houve diferença estatística significativa entre as populações para nenhum dos parâmetros avaliados. Apesar de estatisticamente não diferirem, os valores de diâmetro de entrenó, espessura do córtex, área caulinar

e área do cilindro vascular foram menores para a população 6, indicando a presença de indivíduos de menor porte para essa população. Além disso, esses parâmetros apresentaram fortes correlações, tais como o diâmetro do entrenó e a área do cilindro vascular (r = 0,9216; p = 0,009), a espessura do córtex e a área total do entrenó caulinar (r = 0,46; p = 0,8199) e a área total do caule também apresentou correlação positiva com a área do cilindro vascular (r = 0, 9199; p = 0,01).

Análises de solos

Os resultados obtidos através das análises de solo estão dispostos na Tabela 4. Em relação aos micronutrientes, as maiores variações observadas foram em relação aos teores de Cu, Fe e Mn para a população 6, que foram maiores quando comparados às outras populações.

O pH, tanto em água quanto em cloreto de potássio (KCl), não apresentou grandes variações nos valores entre as populações, assim como os teores de P, Ca, Mg e a saturação por alumínio (m). O teor de K se apresentou levemente maior para a população 6. Já o teor de Al e a acidez potencial (H + Al) foram maiores nas populações 5 e 6, quando comparados às demais. Para a capacidade de troca de cátions (CTC), a população 5 também apresentou os maiores valores, seguida pela população 6. Em relação à saturação da CTC por bases (V), a população 5 apresentou os valores mais baixos.

Em relação à granulometria do solo, a análise mostrou que a população 6 apresentou teor de argila mais elevado que as demais, sendo o solo classificado como argiloso. Os solos das demais populações foram classificados como médio-arenoso (populações 1, 3 e 5), ou como médio-argiloso (populações 2, 4).

Alguns dos parâmetros anatômicos se correlacionaram com os constituintes avaliados para os solos. Entre eles destacam-se o tamanho da nervura central, que foi positivamente correlacionado com o teor de ferro (r = 0,8374; p = 0,038), a espessura do mesofilo que se correlacionou negativamente com o pH em água (r = -0,8178; p = 0,047) e positivamente com o teor de alumínio (r = 0,8811; p = 0,02) e a espessura da cutícula que se correlacionou negativamente também com o pH em água (r = -0,8677; p = 0,025). Em relação aos parâmetros caulinares, houve correlação fortemente negativa entre a espessura do córtex e teores de boro (r = -0,9069; p = 0,013), cobre (r = -0,9519; p = 0,03), ferro (r = -0,8149, p = 0,048), manganês (r = -0,9431; p = 0,005), potássio (r = -0,8964, p = 0,016) e positiva com a quantidade de areia total no solo (r = 0,9474; p = 0,004) e a área total do entrenó e a área do cilindro vascular que foram negativamente correlacionadas ao teor de ferro (r = -0,8740, p = 0,023; r = -0,8473; p = 0,033) e com a quantidade de argila no solo (r = -0,9704, p = 0,001; r = -0,8863, p = 0,019).

Dados ambientais

De acordo com os dados obtidos no WorldClim (Tabela 5), as populações da localidade 2 (populações 4, 5 e 6) apresentam as maiores temperaturas médias anuais, bem como os maiores

valores de precipitação. A altitude também é maior em duas, das três populações da Localidade 2, com exceção da população 6, que tem altitude igual à população 2.

A temperatura média anual se correlacionou positivamente com o índice estomático da face abaxial da folha (r = 0,8607; p = 0,028) e, juntamente com a precipitação anual, apresentou correlação positiva com a espessura da cutícula (r = 0,8305, p = 0,41; r = 0,8880, p = 0,018).

DISCUSSÃO

A nossa hipótese visava testar se as diferentes localidades e, por consequência, as diferentes condições edafo-climáticas às quais as populações estavam inseridas, seriam suficientes para alterar a estrutura anatômica a ponto de as duas localidades exibirem indivíduos com características anatômicas diferentes. O único parâmetro que diferiu estatisticamente entre as duas localidades foi a espessura da cutícula da face adaxial da folha, que foi ligeiramente mais espessa para indivíduos da Localidade 2. A cutícula desempenha papel importante na redução da perda de água, impermeabilização e reflexão de raios solares (Haberlandt 1990; Larcher 2000) e a sua espessura e composição podem ser influenciadas pelos fatores ambientais (Esau 1976; Dickison 2000). Nesse estudo, as cutículas mais espessas se correlacionaram com os locais de maior temperatura média, o que pode ter influenciado nesse resultado. A cutícula em espécies de *Aldama* brasileiras é relativamente delgada (Bombo et al. 2012; Oliveira et al. 2013; Silva et al. 2014) inclusive para *A. grandiflora* aqui avaliada, quando comparadas às espécies sul-americanas não brasileiras (Magenta 2006) e com outras espécies que ocorrem no Cerrado (Bieras & Sajo 2009; Lusa et al. 2014).

Diversos parâmetros ambientais podem afetar o crescimento vegetal, tais como a altitude, que pode influenciar a velocidade de desenvolvimento do vegetal, uma vez que quanto menor a altitude, mais rápida será a velocidade de desenvolvimento (Cordell et al. 1999; Kao & Chang 2001; Kofidis et al. 2003) e, consequentemente, indivíduos encontrados em áreas mais altas geralmente irão apresentar folhas menores (Woodward 1983; Cordell et al. 1998; Kao & Chang 2001). Os parâmetros morfométricos avaliados não apresentaram correlação com a altitude na qual as populações foram amostradas, provavelmente porque a diferença entre a amplitude da altitude entre as localidades não foi tão expressiva, como a abordada por Tiwari et al. (2013), os quais avaliaram as diferenças anatômicas em um gradiente de mais de 1700 m de altitude.

A espécie *Aldama grandiflora* ocorre exclusivamente em áreas abertas de Cerrado (Magenta et al. 2014), e estão sujeitas às condições características desses ambientes, como a alta radiação solar, baixa disponibilidade de nutrientes no solo como cálcio e magnésio e altos teores de alumínio, além de baixa disponibilidade hídrica, principalmente nas camadas superficiais do solo (Ratter et al. 1997), condições às quais podem influenciar a anatomia das espécies vegetais. O índice estomático, que foi um dos parâmetros que diferiu entre as populações e se correlacionou positivamente com a temperatura média anual, apresentou os maiores valores para a população 5, a

qual além de estar sob uma das maiores temperaturas amostradas, também se encontrava na maior altitude. A alta densidade estomática e índice estomático podem ser considerados uma adaptação que aumenta a captação de CO_2 (Dickison 2000), que promove maior saída de vapores de água, aumentado a transpiração e o resfriamento interno da folha (Lima Jr. et al. 2006), importantes em temperaturas mais elevadas; valores altos de índice estomático também são associados à altitudes mais elevadas e, consequentemente, à uma maior incidência luminosa e também, menor teor de oxigênio e CO_2 disponíveis (Apel 1989; Furukawa 1997; Gardoni et al. 2007).

Células epidérmicas mais altas e com paredes espessas são mais aptas a dispersar o excesso de luminosidade, protegendo dessa forma o tecido fotossintético e evitando o superaquecimento foliar (Feller 1996; Evert 2006). Já paredes anticlinais e, ou periclinais com maior espessamento são relatadas com frequência em espécies provenientes de regiões sujeitas a estresse hídrico (Solereder 1908; Metcalfe & Chalk 1979), além de estarem relacionadas à redução da perda de água por transpiração, diminuição do aquecimento no interior do órgão e na manutenção de sua arquitetura e reflexão da maior luminosidade incidente (Dickison 2000; Leite & Scatena 2001). Todos os indivíduos amostrados apresentaram células epidérmicas mais altas na face adaxial da folha e espessamento parietal em pectina (dados não apresentados) na parede periclinal externa de ambas as faces da folha, confirmando a adaptação dessa espécie ao ambiente de campos cerrados, no qual predominantemente ocorre.

A necessidade de nutrientes da camada herbácea, que é um componente essencial da vegetação de savanas, é extremamente baixa, assegurando elevada resiliência ao ecossistema Cerrado depois de distúrbios como o fogo (Batmanian & Haridasan 1985; Villela & Haridasan 1994). As populações aqui amostradas, com exceção da população 4, apresentavam indícios de passagem de fogo recente, comprovada pela presença de bases de ramos carbonizados (Figura 2F) e por restos de fuligem no solo (Figura 2A-C, E-F). Em todas as localidades, os indivíduos de *Aldama grandiflora* eram maioria entre as plantas que já rebrotavam após o fogo, indicando que, apesar da deficiência nutricional nos solos nos quais essas populações se estabeleceram, esses indivíduos estão aptos a sobreviver às condições dos solos do Cerrado (Haridasan 2008).

Além de altos valores de alumínio, manganês e ferro nos solos amostrados, especialmente nas populações 5 e 6, o pH do solo, que influencia a disponibilidade e deficiência dos nutrientes bem como na toxicidade dos mesmos às espécies vegetais (Haridasan 2008), foi ácido (entre 5,2 e 5,4), o que é bem característico de solos de cerrado *sensu stricto* e fisionomias abertas de Cerrado (Lopes & Cox 1977). Apesar de esses elementos serem relatados como tóxicos para muitas plantas, essa toxicidade ocorre apenas em condições de solos ainda mais ácidos (Haridasan 2008). O solo onde foi coletada a população 4 apresentou baixa fertilidade, em especial, quando os nutrientes fósforo (P) e potássio (K) são considerados, e menor capacidade de retenção de água, associado ao alto teor de areia total na camada superficial. O porte relativamente menor dos indivíduos desta população pode estar associado a essas características do solo.

A adaptação ecológica muitas vezes pode estar associada a uma nutrição mineral desfavorável (Metcalfe & Chalk 1983) e Gardoni et al. (2007) já associaram a variação fenotípica encontrada em *Marcetia taxifolia* (A. St.-Hil.) DC. (Melastomataceae) às condições edáficas e ou geológicas, visto que as variações não refletiram as condições geográficas e, ou climáticas. Para a espécie aqui avaliada, alguns dos parâmetros foliares que variaram significativamente entre as populações se correlacionaram com características do solo, tais como o tamanho da nervura central com os teores de ferro e espessura do mesofilo, com os teores de alumínio. Em relação aos parâmetros caulinares, apesar de não diferirem estatisticamente, as variações encontradas entre as populações também refletiram as relações com as condições edáficas, uma vez que o número de camadas corticais nos caules, que indiretamente influencia a área caulinar, se relacionou negativamente com vários dos nutrientes do solo, e ainda, as áreas total e do cilindro vascular, com os teores de ferro.

Portanto, é possível afirmar que mesmo havendo pequenas diferenças morfométricas entre os indivíduos nas diferentes populações avaliadas, em virtude da plasticidade fenotípica dos mesmos, *A. grandiflora* apresenta características anatômicas foliares e caulinares bastante consistentes.

REFERÊNCIAS BIBLIOGRÁFICAS

Apel P. 1989. Influence of CO_2 on stomatal numbers. Biologia Plantarum 31:72-74.

Baas P. 1982. Systematic, phylogenetic, and ecological wood anatomy - History and perspectives. *In* New perspectives in wood anatomy (P Baas, N Martinus) Junk Publishing, Hague, p. 23-58.

Batmanian GJ, Haridasan M. 1985. Primary production and accumulation of nutrients by the ground layer community of cerrado vegetation of central Brazil. Plant and Soil 88: 437-440.

Bieras AC, Sajo MG. 2009. Leaf structure of the cerrado (Brazilian savanna) woody species. Trees 23: 451-471.

Bombo AB, Oliveira TS, Oliveira ASS, Rehder VLG, Magenta MAG, Appezzato-da-Glória B. 2012. Anatomy and essential oils from aerial organs in three species of *Aldama* (Asteraceae-Heliantheae) that have a difficult delimitation. Australian Journal of Botany 60: 632-642.

Bombo AB, Oliveira TS, Oliveira ASS, Rehder VLG, Appezzato-da-Glória B. 2014. Anatomy and essential oil composition of the underground systems of three species of *Aldama* La Llave (Asteraceae): Journal of the Torrey Botanical Society 141: 115-125.

Bombo AB, Filartiga ALP, Appezzato-da-Glória B. Em preparação. Secretory structures in *Aldama* La Llave species: morphology, histochemistry and composition of essential oil. Manuscrito em preparação, capítulo 3 da tese.

Bukatsch F. 1972. Bermerkungen zur Doppelfarbung Astrablau-Safranin: Mikrokosmos 61: 1.

Canales M, Hernández T, Rodríguez-Monroy MA, Jiménez-Estrada M, Flores CM, Hernández LB, Gijón IC, Quiroz S, García AM, Ávila G. 2008.Antimicrobial activity of the extracts and essential oil of *Viguiera dentata*. Pharmaceutical Biology 46: 719-723.

Carlquist, S. 1988. Comparative Wood Anatomy. Springer-Verlag, Berlin.

Cordell S, Goldstein G, Mueller-Dombois D, Weeb D, Vitousek PM. 1998. Physiological and morphological variation in *Metrosideros polymorpha*, a dominant Hawaiian tree species, along an altitudinal gradient: the role of phenotypic plasticity. Oecologia 113: 188-196.

Cronquist A. 1981. An integrated system of classification of flowering plants. Columbia University Press, New York.

Cutter EG. 1986. Anatomia vegetal. Parte I Células e tecidos. 2.ed. Roca, São Paulo.

Dickison WC. 2000. Integrative Plant Anatomy. Elsevier, San Diego.

Esau K. 1976. Anatomia das plantas com sementes. Edgard Blücher, São Paulo.

Evert RF. 2006. Esau's plant anatomy: meristems, cells and tissue of the plant body: their structure, function and development. 3rd ed., Wiley, New Jersey.

Fahn A. 1986. Structural and functional properties of trichomes of xeromorphic leaves. Annals of Botany 57: 631-637.

Feller IC. 1996. Effects of nutrient enrichment on leaf anatomy of dwarf *Rhizophora mangle* L. (red mangrove). Biotropica 28(1): 13-22.

Furukawa A. 1997. Stomatal frequency of *Quercus myrsinaefolia* grown under different irradiances.Photosynthetica 34:195-199.

Gardoni LCP, Isaias RMS, Vale FHA. 2007. Morfologia e anatomia foliar de três morfotipos de *Marcetia taxifolia* (A. St.-Hil.) DC. (Melastomataceae) na Serra do Cipó, MG. Revista Brasileira de Botânica 30(3): 487-500.

Givnish TJ. 1984. Leaf and canopy adaptations in tropical forests. *In* Physiological ecology of plants in the wet tropics (E Medina, HA Mooney, C Vasques-Yanes, eds.). Dr. W. Junk Publishers, The Hague, p.51-84.

Haberlandt G. 1990. Physio1ogica1 plant anatomy. 2nd ed., Today & Tomorrow's printers & publishers, New Delhi.

Haridasan M. 2008. Nutritional adaptations of native plants of the cerrado biome in acid soils. Brazilian Journal of Plant Physiology 20(3): 183-195.

Jensen W. 1962. Botanical histochemistry, principles and practice. W. H. Freeman, San Francisco.

Johansen D. 1940. Plant microtechnique. McGraw-Hill Book Co. Inc., New York.

Kao WY, Chang KW. 2001. Altitudinal trends in photosynthetic rate and leaf characteristics of *Miscanthus* populations from central Taiwan. Australian Journal of Botany 49: 509-514.

Kofidis G, Bosabadilis AM, Moustakas M. 2003. Contemporary seasonal and altitudinal variations of leaf structural features in oregano (*Origanum vulgare* L.). Annals of Botany 92: 635-645.

Kuniyoshi YS. 1993. Aspectos morfo-anatômicos do caule, raíz e folha de *Tabebuia cassinoides* (Lam.) DC. (Bignoniaceae) em diferentes fases sucessionais no litoral do Paraná. Tese de doutorado, Universidade Federal do Paraná, Curitiba.

Larcher, W. 2000. Ecofisiologia vegetal. Rima, São Carlos.

Leite, A.M., Lima, E.O., Souza, E.L., Diniz, M.F.F.M., Trajano, V.N., Medeiros, I.A., 2007. Inhibitory effect of β-pinene, α-pinene and eugenol on the growth of potential infectious endocarditis causing Gram-positive bacteria. Braz. J. Pharm. Sci. 43, 121-126.

Leite KRB, Scatena VL. 2001. Anatomia do segmento foliar de espécies de *SyagrusMart*. (Arecaceae) da Chapada Diamatina, Bahia, Brasil. Sitientibus, série Ciências Biológicas 1: 3-14.

Lima Jr EC, Alvarenga AA, Castro VV, Barbosa JPRA. 2006. Aspectos fisioanatômicos de plantas jovens de *Cupania vernalis* Camb. Submetidas a diferentes níveis de sombreamento. Revista Árvore 30 (1): 33-41.

Lopes AJ, Cox FR. 1977. A survey of the fertility status of surface soils under cerrado vegetation of Brazil. Soil Science Society of America Journal 41: 752-757.

Lusa M, Appezzato-da-Glória B, Loeuille B, Bartoli G, Ciccarelli D. 2014. Functional groups in Lychnophorinae (Asteraceae: Vernonieae) based on morphological and anatomical traits: Australian Journal of Botany 62: 150-163.

Magenta MAG. 2006. *Viguiera* Kunth (Asteraceae, Heliantheae) na América do Sul e sistemática das espécies do Brasil. Universidade de São Paulo, São Paulo.

Magenta MAG, Pirani JR. 2014. Novidades taxonômicas em *Aldama* (Asteraceae-Heliantheae): Rodriguésia 65(1): 175-192.

Magenta MAG, Pirani JR, Mondin CA. 2010. Novos táxons e combinações em *Viguiera* (Asteraceae - Heliantheae). Rodriguésia 61: 1-11.

Metcalfe CR, Chalk L. 1979. Anatomy of the Dicotyledons, Systematic Anatomy of the leaf and stem. v.1, Clarendon Press, Oxford.

Metcalfe CR, Chalk L. 1983. Anatomy of the dicotyledons. 2nd ed., v.2, Clarendon Press, Oxford.

Oliveira TS, Bombo AB, Appezzato-da-Glória B. 2013. Anatomy of vegetative organs with an emphasis on the secretory structures of two species of *Aldama* (Asteraceae – Heliantheae): Botany 91(3): 335-342.

Panero JL, Crozier BS. 2012. Asteraceae. Sunflower, daisies. Version 27, January 2012. The Tree of Life Web Project, http://tolweb.org/ (acessed 2016 Apr 28).

Rasband WS. 2006. Image J, U. S. Nacional Institute of Health, Bethesda, Maryland. http://rsb.info.nih.gov/ij.

Ratter JA, Askew GD, Montgomery RF, Gifford DR. 1977. Observações adicionais sobre o cerradão de solos mesotróficos no Brasil Central. *In* IV Simpósio Sobre o Cerrado (Ferri MG ed.), EDUSP, São Paulo, p. 306-316.

Sakai W. 1973. Simple method for differential staining of paraffin embedded plant material using toluidine blue O. Stain Technology 48: 247-249.

Santos, M.R.V., Moreira, F.V., Fraga, B.P., Souza, D.P., Bonjardim, L.R., Quintans-Junior, L.J., 2011. Cardiovascular effects of monoterpenes: a review. Rev. Bras. Farmacog.21, 764-771.

Scheiner SM. 1993. Genetics and evolution of phenotypic plasticity. Annual Review of Ecology, Evolution, and Systematics 24: 35-68.

Schilling E, Panero J. 2011. A revised classification of subtribe Helianthinae (Asteraceae: Heliantheae) II. Derived lineages: Botanical Journal of the Linnean Society 167: 311-331.

Silva EMS, Hayashi AH, Appezzato-da-Glória B. 2014. Anatomy of vegetative organs in *Aldama tenuifolia* and *A. kunthiana* (Asteraceae: Heliantheae). Brazilian Journal of Botany 37: 505-517.

Solereder H. 1908. Systematic anatomy of the dicotyledons: A handbook for laboratories of pure and applied botany. v. 1, Clarendon Press, Oxford.

Statsoft Inc. 2007. STATISTICA for windows. Statsoft Inc. 2300, East 14th Street, Tulsa, OK. AStA 91: 339–341.

Stearns SC. 1989. The evolutionary significance of phenotypic plasticity. BioScience 39: 436-445.

Stevens PF. 2001. Angiosperm Phylogeny Website. v.8, Junho de 2007. http://www.mobot.org/MOBOT/research/APweb/ (accessed 2016 Apr 28)

Tiwari SP, Kumar P, Yadav D, Chauhan DK. 2013. Comparative morphological, epidermal, and anatomical studies of *Pinus roxburghii* needles at different altitudes in the North-West Indian Himalayas. Turkish Journal of Botany 37: 65-73.

Villela DMV, Haridasan M. 1994. Response of the ground layer community of a cerrado vegetation in central Brazil to liming and irrigations Plant Soil 163: 25-31.

Woodward FI. 1983. The significance of interspecific differences in specific leaf area to the growth of selected herbaceous species from different altitudes. New Phytologist 95: 313-323.

Yaltirik F. 1970. Comparison of anatomical characteristics of wood in Turkish maples with relation to the humidity of the sites. Journal of the Institute of Wood Science 25(5): 43-8.

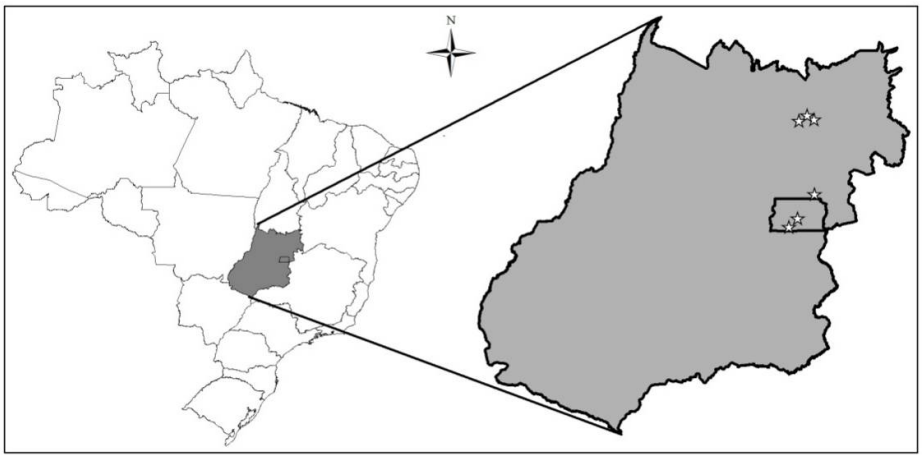

Figura 1. Localização das populações de *Aldama grandiflora* amostradas, no estado de Goiás.

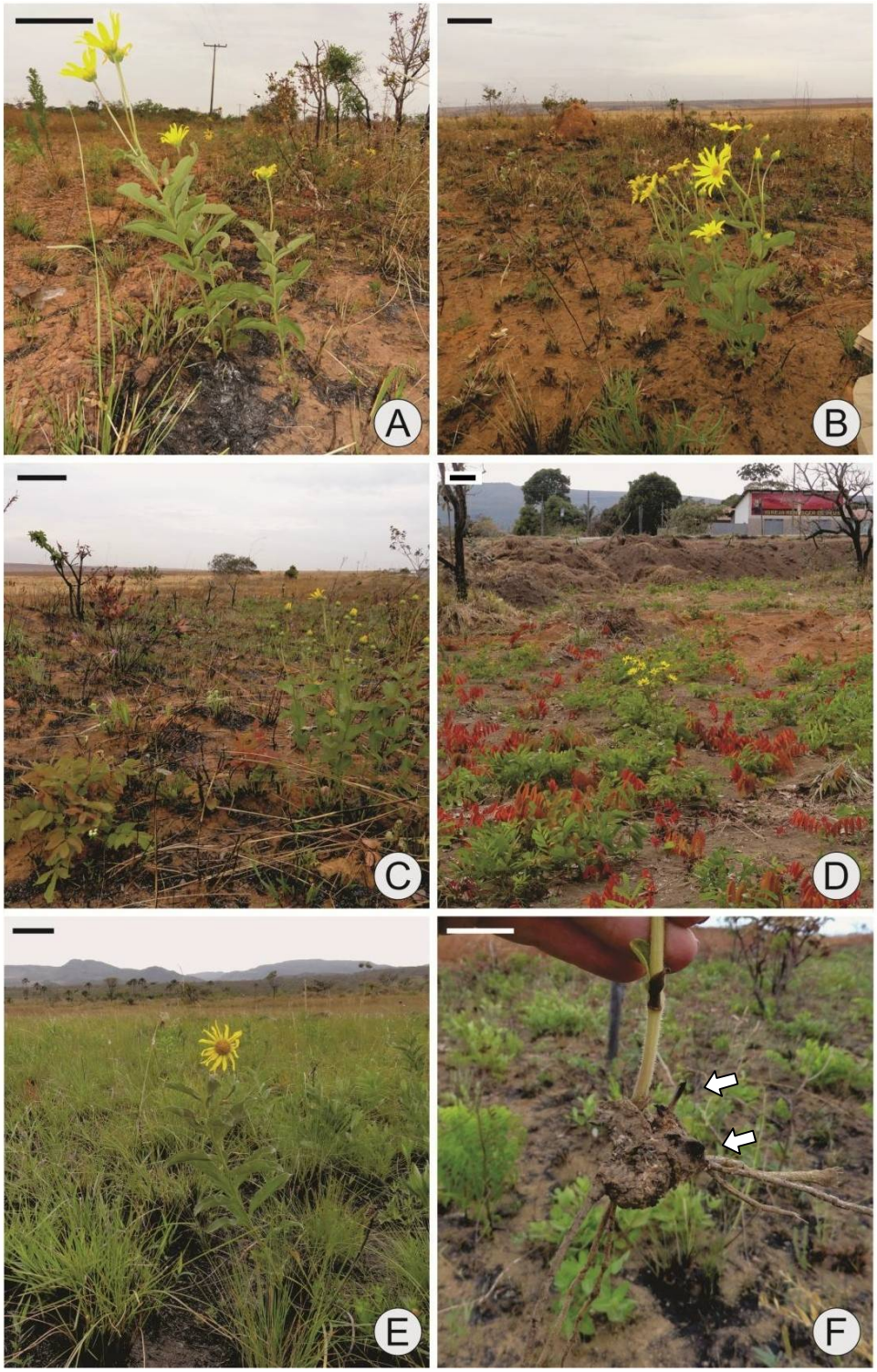

Figura 2. Visão geral nocampodas populações de *Aldama grandiflora* amostradas. A. População 1, Brasília/DF. B. População 2, Brasília/DF. C. População 3, Planaltina/GO. D. População 4, Alto ParaísodeGoiás/GO. E. População 5, PARNA Chapada dos Veadeiros —Alto Paraíso de Goiás/GO (Mulungu). F. População 6, Alto Paraíso de Goiás/GO (Vale da Lua). Notar as bases dos ramos carbonizados (setas) e o detalhe do sistema subterrâneo. Barras: A-C,E =10cm; D = 20cm; F = 2cm.

Tabela 1. Local, altitude da estação meteorológica mais próxima, nome da estação e coordenadas geográficas das populações coletadas de *Aldama grandiflora*. Fonte dos dados estações meteorológicas: INMET.

Pop	Local (Cidade, Estado)	Altitude	Nome da estação	Coordenadas geográficas	Voucher
1	Brasília/DF	1152 m	Faculdade da Terra	S 15°58'51,2" W 047°56'54,9"	ESA134833
2	Brasília/DF	1160 m	Brasília	S 15°50'05,6" W 047°48'13,1"	ESA134834
3	Planaltina/GO	1200 m	Águas Emendadas	S 15°25'27,3" W 047°31'03,3"	ESA134835
4	Alto Paraíso de Goiás/GO	1240 m	Alto Paraíso de Goiás	S 14°08'34,9" W 047°31'19,8"	ESA134836
5	PARNA Chapada dos Veadeiros – Alto Paraíso de Goiás/GO (Mulungu)	1205 m	Alto Paraíso de Goiás	S 14°04'24,0" W 047°38'09,7"	ESA134837
6	Alto Paraíso de Goiás/GO (Vale da Lua)	1040 m	Alto Paraíso de Goiás	S 14°10'06,2" W 047°46'29,8"	ESA134838

Tabela 2. Parâmetros anatômicos das folhas (média ± desvio padrão) e resultado do Teste de Tukey para as seis populações de *Aldama grandiflora*. Diferentes letras em uma mesma linha representam diferenças significativas entre as populações ($p < 0,05$).

	Localidade 1						**Localidade 2**					
Parâmetros	**1**		**2**		**3**		**4**		**5**		**6**	
Área foliar (cm^2)	36,32 ± 10,56	a	23,79 ± 6,81	a	29,11 ± 7,67	a	23,49 ± 10,585	a	30,30 ± 15,579	a	26,457 ± 11,096	a
Índice estomático face adaxial	16,13 ± 2,1	ab	17,64 ± 2,42	a	16,04 ± 2,11	ab	16,90 ± 1,223	ab	17,75 ± 1,737	a	14,76 ± 1,566	b
Índice estomático face abaxial	17,06 ± 2,06	b	19,25 ± 2,66	ab	20,15 ± 2,44	ab	18,96 ± 3,140	ab	20,97 ± 2,461	a	21,34 ± 2,59	a
Espessura da nervura central (µm)	1421,21 ± 191,38	ab	1205,27 ± 141,51	b	1331,70 ± 157,03	ab	1381,69 ± 195,855	ab	1368,0 ± 172,778	ab	1525,316 ± 162,968	a
Número de canais na nervura central	5,74 ± 1,23	a	4,82 ± 0,94	a	4,72 1,27	ab	3,36 ± 0,771	b	4,52 ± 1,136	ab	5,36 ± 1,349	a
Espessura mesofilo (µm)	301,93 ± 43,35	ab	263,81 ± 24,22	b	288,73 ± 28,81	b	300,82 ± 52,681	ab	348,22 ± 34,733	a	313,961 ± 28,884	ab
Número de canais no mesofilo	1 ± 0	a	1,1 ± 0,31	a	1,2 ± 0,38	a	1 ± 0	a	1 ± 0	a	1,2 ± 0,422	a
Espessura epiderme adaxial (µm)	38,7 ± 4,62	a	36,04 ± 3,73	a	37,81 ± 4,18	a	36,15 ± 3,905	a	40,06 ± 5,118	a	39,60 ± 6,748	a
Espessura epiderme abaxial (µm)	23,16 ± 4,58	a	22,68 ± 3,69	a	20,17 ± 3,52	a	19,74 ± 4,513	a	23,08 ± 4,098	a	21,56 ± 4,809	a
Espessura parede periclinal externa face adaxial (µm)	6,83 ± 1,05	a	5,66 ± 0,57	a	5,92 ± 0,52	a	6,09 ± 1,209	a	6,50 ± 1,129	a	6,67 ± 1,043	a
Espessura parede periclinal externa face abaxial (µm)	7,29 ± 1,81	a	5,96 ± 1,0	a	7,29 ± 1,19	a	6,65 ± 1,940	a	7,52 ± 1,795	a	7,25 ± 1,540	a
Espessura cutícula face adaxial (µm)	1,92 ± 017	b	2,03 ± 0,28	b	2,05 ± 0,19	b	3,32 ± 0,404	a	3,80 ± 1,157	a	3,39 ± 0,568	a

Tabela 3. Parâmetros anatômicos caulinares (média ± desvio padrão) e resultado do Teste de Tukey para as seis populações de *Aldama grandiflora*. Diferentes letras em uma mesma linha representam diferenças significativas entre as populações ($p < 0,05$).

	Localidade 1			**Localidade 2**		
Parâmetros	**1**	**2**	**3**	**4**	**5**	**6**
Diâmetro do entrenó (cm)	0,47 ± 0,11 a	0,50 ± 0,1 a	0,52 ± 0,1 a	0,45 ± 0,07 a	0,59 ± 0,17 a	0,42 ± 0,03 a
Nº camadas no córtex	14,60 ± 3,14 a	15,33 ± 2,58 a	14,20 ± 1,4 a	15,33 ± 2,81 a	11,20 ± 0,35 a	12,07 ± 1,50 a
Espessura do córtex (mm)	0,33 ± 0,12 a	0,32 ± 0,06 a	0,35 ± 0,05 a	0,33 ± 0,03 a	0,32 ± 0,06 a	0,25 ± 0,05 a
Nº canais no córtex	27,73 ± 1,6 a	28,93 ± 1,22 a	34,60 ± 3,56 a	34,20 ± 3,7 a	24,93 ± 4,6 a	26,40 ± 5,6 a
Área do caule (mm^2)	22,30 ± 9,68 a	20,2 ± 9,68 a	22,73 ± 8,20 a	18,13 ± 5,78 a	22,39 ± 9,35 a	15,50 ± 1,74 a
Área do cilindro vascular (mm^2)	16,1 ± 6,07 a	15,83 ± 8,11 a	16,07 ± 6,82 a	14,13 ± 5,2 a	18,07 ± 7,79 a	12,17 ± 0,85 a

Tabela 4. Análises química e física dos solos provenientes das seis populações de *Aldama grandiflora* estudadas. Micronutrientes no solo, em $mg.dm^{-3}$. SB: Soma de bases trocáveis; CTC: Capacidade de troca de cátions; V: Saturação da CTC por bases; m: Saturação por alumínio.

	Localidade 1			**Localidade 2**		
Características / Nutrientes	**1**	**2**	**3**	**4**	**5**	**6**
B ($mg.dm^{-3}$)	< 0,12	< 0,12	< 0,12	< 0,12	0,13	0,14
Cu ($mg.dm^{-3}$)	< 0,3	< 0,3	< 0,3	< 0,3	< 0,3	0,9
Fe ($mg.dm^{-3}$)	35	25	31	52	35	73
Mn ($mg.dm^{-3}$)	4,2	0,8	0,9	2,1	0,6	37,1
Zn ($mg.dm^{-3}$)	< 0,4	< 0,4	< 0,4	< 0,4	< 0,4	< 0,4
pH H_2O	5,3	5,4	5,3	5,2	5,2	5,2
pHKCl	4	4,1	4,1	4	4,1	4
P ($mg.Kg^{-1}$)	1	1	1	< 1	2	1
K ($mmolc.Kg^{-1}$)	1,1	1,1	1	1,1	2	2,7
Ca ($mmolc.Kg^{-1}$)	< 2	< 2	3	< 2	< 2	< 2
Mg ($mmolc.Kg^{-1}$)	< 1	< 1	1	1	1	2
Al ($mmolc.Kg^{-1}$)	15	15	14	17	41	24
H + Al ($mmolc.Kg^{-1}$)	31	52	27	34	151	77
SB ($mmolc.Kg^{-1}$)	2,3	2,2	4,5	3	3,4	5,4
CTC ($mmolc.Kg^{-1}$)	33,3	54,3	31,1	36,7	154	82,5
V (%)	7	4	14	8	2	7
m (%)	87	87	75	85	92	82
Areia total ($g.kg^{-1}$)	709	611	772	630	485	268
Silte ($g.kg^{-1}$)	89	81	76	65	327	370
Argila ($g.kg^{-1}$)	202	208	151	305	188	362
Classe de textura	média arenosa	média argilosa	média arenosa	média argilosa	média arenosa	argilosa

Tabela 5. Altitude (m), temperatura média anual (° C), precipitação anual (mm) nas seis localidades amostradas. Dados retirados de WorldClim (http://www.worldclim.org/).

	Localidade 1			**Localidade 2**		
População	**1**	**2**	**3**	**4**	**5**	**6**
Altitude	1169	1079	1155	1243	1206	1079
Temperatura média anual	20,5	21,0	21,1	21,5	21,7	22,3
Precipitação anual	1593	1627	1429	1805	1839	1780

CAPÍTULO III

Secretory structures in *Aldama* La Llave species: morphology, histochemistry and composition of essential oils

(Segundo normas *Flora)*

Secretory structures in *Aldama* La Llave species: morphology, histochemistry and composition of essential oils

Aline Bertolosi Bombo, Arinawa Liz del Prado Filartiga, Vera Lucia Garcia, Beatriz Appezzato-da-Glória

ABSTRACT

Aldama representatives are particularly noteworthy for producing essential oils, and the chemical composition identification of these oils can help in the search for possible bioactive compounds, and provide useful information for taxonomic studies. A correct description of the occurrence and positioning of the secretory structures related to the production of these metabolites in the plant body could provide a basis for future studies on biological and pharmacological activity, indicating which organ merits further investigation. The four *Aldama* species investigated here were chosen due to their aromatic and resiniferous potential and because they are morphologically very similar. They were investigated phytochemically and their secretory structures were identified. Four types of secretory structures were found for the species herein: hydathodes, glandular trichomes, canals and cavities. Except for the hydathodes, all of them are responsible for the essential oil production. The highest yields of essential oils were obtained the underground organs. Seventy-nine compounds were identified for the four *Aldama* species, whereas 13 compounds occurred in the essential oils from all species and some of them were unique to a given species. The chemical composition of the four species, described herein for the first time, can be used for identification purposes and, also reveals several compounds of proven biological activity, confirming the potential usefulness of *Aldama* species.

1. Introduction

Aldama La Llave, a genus of the Asteraceae family with exclusively American representatives (Schillinget al., 2000) and previously included in the *Viguiera* Kunth genus (Magenta and Pirani, 2014), has shown great promise for the production of potentially active secondary compounds (Marquinaet al., 2001; Canales et al., 2008; Bombo et al. 2015). However, chemical studies on the genus have so far mainly focused on the occurrence of diterpenes (Ambrosioet al., 2004; Porto et al., 2009; Carvalho et al., 2011) and sesquiterpene lactones (Springet al., 2003; Valerio et al., 2007; Arakawa et al., 2008; Nicolete et al., 2009).

Several aspects of the plant's secretory structures have been widely studied due to the importance of the substances they can produce. Many of the natural chemicals produced by these structures are commercially and ecologically important because of the role they play in attracting pollinators, protecting cultivated plants from herbivore attacks and, in particular, because of their importance in the pharmaceutical industry (Mccaskill and Croteau, 1999; Ploset al., 2011). Several types of secretory structures have already been reported for the *Aldama* genus, such as canals and cavities, idioblasts, hydathodes and glandular trichomes (Castro et al., 1997; Bombo et al., 2012; Oliveira et al., 2013a; Bombo et al., 2014; Silva et al., 2014).

Aldama representatives are particularly noteworthy for producing essential oils, and it has been demonstrated that identifying the chemical composition of these oils can help in the search for possible bioactive compounds (Appezzato-da-Glóriaet al., 2012; Rehder et al., 2012; Oliveira et al., 2013b), and provide useful information for taxonomic studies (Bomboet al., 2012; Oliveira et al., 2013a; Bombo et al., 2014; Silva et al., 2014). Moreover, a correct description of the occurrence and positioning of the secretory structures related to the production of these metabolites in the plant body could provide a basis for future studies on biological and pharmacological activity, indicating which organ merits further investigation.

The Brazilian *Aldama* genus comprise 35 species, 17 of which are endemic (Magenta and Pirani, 2014). *Aldama bakeriana* S.F. Blake E.E.Schill. & Panero, *A. discolor* (Baker) E.E.Schill. & Panero, *A. grandiflora* (Gardner) E.E.Schill. & Panero and *A. squalida* (S. Moore) E.E.Schill. & Panero were chosen for this study due to their aromatic and resiniferous potential and because they are morphologically very similar (Magenta and Pirani, 2014).

We therefore decided to perform a comprehensive phytochemical study on the vegetative organs of these four species of *Aldama*, analyzing the composition of their essential oils. To complete the study, a morphological and histochemical survey of the plants' secretory structures was conducted, aimed in particular at locating secondary metabolite biosynthesis sites and focusing especially on the essential oil site of synthesis.

2. Material and methods

2.1 Plant material

Samples of the four *Aldama* species were collected in different populations during 2012. *A grandiflora* was collected in Brasília and Alto Paraíso de Goiás (Brazilian state of Goias) (ESA123037, ESA123038, ESA123040), *A. bakeriana* in Alpinópolis, Divinópolis and Oliveira (state of Minas Gerais) (ESA123041, ESA123042, ESA123043), *A. discolor* in Pedregulho, Sacramento and Uberlândia, (state of Minas Gerais) (ESA123044, ESA123045, ESA123046) and *A. squalida* in Campos Grande and Ribas do Rio Parto, (state of Mato Grosso do Sul) (SPF 215969, SPF 215970).The species were identified by a Brazilian specialist and the

herbarium material was registered and incorporated into the collections of the Luiz de Queiroz School of Agriculture, University of Sao Paulo (ESA herbarium) and the University of Sao Paulo herbarium (SPF).

2.2 Anatomical analysis

Cross-sections and longitudinal sections of samples from leaves, aerial and underground stems, and roots selected from each species were analyzed. The analysis was conducted on fully expanded leaves (at the apex, middle of the blade and leaf margin), the youngest and the oldest (nearer the ground) internodes of aerial stems, different regions of the underground stem and non-tuberized and tuberized portions of the roots.

All these samples were fixed in FAA 50 (formaldehyde, acetic acid and 50 % ethanol) (Johansen, 1940), placed in a vacuum pump to remove air from the tissue, dehydrated in a graded ethanol series and kept in 70 % ethanol. To prepare the slides, samples were dehydrated in a graded ethanol series and embedded in plastic resin (Leica Historesin®). The blocks were then sectioned (5–8 µm thick) using a Leica RM 2045 rotary microtome. The sections were stained with 0.05 % toluidine blue O in citrate–phosphate buffer, pH 4.5 (Sakai, 2002) and mounted in Entellan® synthetic resin (Merck, Darmstadt, Germany). Some samples, used to histochemical analysis were also fixed in NBF (neutral buffered formalin) (Lillie, 1948 *in* Clarke, 1973).

Sections of fixed material (FAA or NBF), some embedded in historesin, were used for histochemical analysis. The following reagents and stains were used for detection: Sudan IV (Jensen, 1962) and neutral red (under UV light – Leica® I3 or N2.1 filter) (Kirk, 1970) for total lipids; Nile blue sulfate for neutral lipids (Cain, 1989); NADI reagent for terpenes (David and Carde, 1990); PAS reaction (periodic acid-Schiff) for total polysaccharides (Mcmanus, 1948); methylene blue for basic mucilage (Johansen, 1940); ruthenium red for mucilage (Gregory and Baas 1989) and pectic substances (Johansen 1940); Wagner reagent for alkaloids (Furr and Mahlberg, 1981); zinc-chloride iodide for starch grains (Strasburger, 1913); Calcofluor White M2R for cellulose (Hughes and Mccully, 1975) (under UV light – Leica® A filter) and ferric chloride for phenolic compounds (Johansen, 1940). Control sections were prepared as standard procedure to confirm the histochemical tests.

Photomicrographs were taken with a Leica DMLB microscope and Leica DFC310Fx camera. LAS 4.0 software (Leica) was used for image analysis.

Leaf and stem surfaces were analyzed under a scanning electron microscope using samples previously fixed in Karnovsky solution (Karnovsky, 1965), dehydrated in a graded ethanol series and subjected to critical-point drying with CO_2 (Horridge and Tamm, 1969), mounted on aluminum stubs and coated with gold (30–40 nm) using a Balzers SCD 050 sputter-coater. The photomicrographs were obtained using a LEO 435 VP SEM (Zeiss, Oberkochen,

Germany) operating at 20 kV, and scale bars were printed directly onto the electron micrographs produced.

2.3 Essential-oil extraction and analysis

Four extractions were performed for each species (one for each organ: leaves, aerial stems, underground stems and roots) using fresh material that was fractionated to optimize the process. The amount of fresh matter was determined according to the availability of the plant material in each sampled population during field collection (Table 1). The essential oils were obtained by hydrodistillation for 3 hours in a Clevenger-type apparatus. This operation was triplicated, with each population representing one repetition one repetition. The only exception was *A. squalida*, with samples from only two populations for each organ.

Samples of essential oils were analyzed by gas chromatography coupled with mass spectrometry (GC–MS) using an HP 5890 series II chromatograph (Hewlett-Packard, Palo Alto, CA, USA), Hewlett-Packard 5971 mass-selective detector, split/splitless injector, and HP-5 capillary column (25 m x 0.20 mm x 0.33 mm). Temperatures were as follows: injector = 220 °C; detector = 280 °C; and column = 60 °C, gradually raised by 3°C.min^{-1} to 240 °C and kept for 7 min at this temperature; the flow rate of carrier gas (super-dry He) was 1.0 mL.min^{-1}. Essential oils were also analyzed by gas chromatography coupled with flame ionization detection (GC-FID) using a Thermo Scientific TRACE™ CG-ULTRA Gas Chromatograph with flame ionization detector (FID); AS 3000 auto-sampler, split/splitless injector, and an HP-5 (25 m x 0.20 mm x 0.33 µm) capillary column. Temperatures were as follows: injector = 220 °C; detector = 280 °C; and column = 60 °C, gradually raised by 3°C.min^{-1} to 240 °C and kept for 7 min at this temperature; the super-dry He carrier gas flow rate was 1.0 mL.min^{-1}. All samples were dissolved in ethyl acetate at a concentration of 20 mg.mL^{-1}.

Compounds were identified based on retention time data obtained from GC-FID, and the mass spectra obtained from GC-MS. Mass spectra were compared with data from the NIST 11 Mass Spectral Library (Fabrication Varian Inc.). Arithmetic indexes (AI) were calculated using the Van den Dool Kratz equation and co-injection patterns for hydrocarbons, as well as the data described by Adams (2007).

2.4 Statistical analysis

In order to determine whether the differences among the essential oil yields from different organs in a same species were significant, essential oil yield values were subjected to variance analysis and means compared using the Tukey test ($p < 0.05$) run on Statistica 8 software (Statsoft Inc., 2007).

Clustering and ordination analysis were performed to verify chemical similarity among species. To do this, a compound presence/absence matrix for the different organs and populations of each species was prepared, based on Table 2. Compounds that could not be

structurally identified and are therefore not shown in Table 2 were also taken into account and identified by the corresponding calculated arithmetic indices. The hierarchical clustering method (UPGMA, Unweighted Pair Group Method using Arithmetic Mean – Sneath and Sokal, 1973) was used, based on the distance calculated by the Jaccard index. The similarity among the EOs from these species was also investigated using Principal Coordinate Analysis (PCoA), an ordination method that, based on any distance measurement, allows the position of the samples to be visualized in relation to each other, using a reduced number of dimensions (Gotelli and Ellison, 2011). These analyses were run on FITOPAC 2.1.2.85 software (Shepherd, 2010).

3. Results

3.1 Secretory structures: anatomy and distribution

Anatomical analysis revealed the presence of hydathodes, glandular trichomes, secretory canals and cavities in all species studied. All four species exhibited hydathodes on the leaf margin (Figure 1A) and apex, and linear glandular trichomes (LGT) (Figure 1A-C) on the leaf surface (Figure 1A,C) and the youngest portions of the aerial stem (Figure 1B). Capitate glandular trichomes were observed only in *Aldama discolor* and *A. grandiflora*, on the abaxial leaf epidermis (Figure 1B,E) and, as for LGTs, on the youngest portions of the aerial stem (Figure 1C).

Secretory canals were found extensively and consistently in the organs and species analyzed. However, they varied in lumen size (smallest in *Aldama bakeriana* and largest in *A. grandiflora*). In the leaves, canals were found in the ground parenchyma of the midrib (Figure 1F), in the phloem in the vascular bundle of the midrib (Figure 1G), and in the sheath extensions of lateral bundles (Figure 1G). In the stems, secretory canals (Figure 1H) were observed in the cortical region (Figure 1I), the primary phloem of the vascular cylinder (Figure 1I, inset) and the perimedullary region (Figure 1I) of all four species. They were also observed in the secondary phloem, but only in *A. squalida*. In the roots, they occurred in the cortical regions (Figure 1J) of all species and in the secondary phloem (Figure 1K) of *A. discolor*,*A. grandiflora* and *A. squalida*. Secretory cavities were found in all species, but only in the tuberized portions of the roots, and located under the secondary protective tissue in *A. bakeriana* and in the parenchymatic pith of all four species (Figure 1L). In underground stems, canals were found in the cortical regions (Figure 1M), the secondary phloem (Figure 1M-N) and the medullary portions of the stem axis (Figure 1O). The underground organs of *A. grandiflora* exhibited a higher number of canals with larger lumina than the other species.

3.2 Histochemical screening

Lipophilic substances, mucilage, pectic substances, phenolic compounds and polysaccharides were identified by histochemical screening. There was also a positive reaction to alkaloids, but only for the secretory structures found in the underground organs of *Aldama bakeriana*.

In regard to trichomes, the content of the distal cells of LGTs (Figure 2A-F) reacted positively for pectic substances when stained with methylene blue (Figure 2B) and ruthenium red, and in the PAS reaction (Figure 2C). The same cells had walls containing cellulose, as confirmed by Calcofluor White (Figure 2D). The median cells of LGTs accumulated phenolic compounds, shown by the response to ferric chloride (Figure 2E), and the basal cells reacted positively for lipophilic substances (Figure 2F).

CGTs (Figure 2G-I) reacted positively for lipophilic substances only (Figure 2H), characterized by acid (Nile blue sulfate), neutral lipids (neutral red) and terpenes (NADI reagent) (Figure 2I). In addition to the secretory structures, droplets of lipophilic nature were found in the mesophyll of all four species (Figures 2J-K).

The exudate of leaf secretory canals was transparent in non-stained sections (Figure 2L) and reacted positively for pectic and mucilaginous substances (Figure 2M-N), while the secretory epithelial cells reacted positively for lipophilic substances (Figure 2K,O). In the aerial stems (Figure 2P), roots (Figure 2Q-S) and underground stems (Figures 2T-V), the exudate in secretory canals and cavities reacted positively for lipophilic substances, especially terpenes (Figure 2T), and there was a positive reaction for alkaloids, but only in the secretory structures found in the underground organs of *Aldama bakeriana*, (Figure 2S,U). The secretory structures observed in the underground stems of all four species, and especially those located in the outermost portions, were found in some cases to exhibit a yellowish exudate of a resinous consistency (Figure 2V).

3.3 Essential oil yield and chemical composition

The essential oil (EO) yield varied from one species to another, and from one organ to another within the same species (Figure 3). Higher yield values were found for the leaves, roots and underground stems of *Aldama grandiflora*, with the lowest yield values shown by *A. bakeriana*. Moreover, with the exception of *A. discolor*, higher oil yield values were observed for roots and/or underground stems.

GC-MS analysis identified 79 compounds in total for the four *Aldama* species (Table 2). The data shown in Table 2 were obtained from the mean values for each species in the populations analyzed. The standard deviation was quite high for some compounds, due to the discrepancy in relative percentage values for the compound concerned from one population

to another, indicative of wide individual variability in the chemical composition of the essential oil.

For *Aldama grandiflora* and *A. squalida*, the main components of essential oils from underground organs were monoterpenes, whereas the main components from EOs produced by aerial organs were sesquiterpenes. In *A. discolor*, EO chemical composition was the most diversified in terms of the number of different compounds, and well represented by diterpenes. For *A. bakeriana*, diterpenes were also quite common, mainly in EOs from underground organs. However, they were not identifiable and are therefore not shown in Table 2. The presence of these diterpenes was confirmed by the peak retention time and molecular weights (MW) of the compounds (ranging from 272 to 302). The diterpenes found in these samples could not be identified since they were in short supply in the NiST library of the equipment, as there was no hydrocarbon availability of patterns for co-injection and comparison.

Among the 79 compounds identified (Table 2), 13 were found in EOs from all four species (Table 3) in at least one organ, especially β-Pinene and Germacrene D (found in EOs from the aerial organs of *A. bakeriana* and in EOs from aerial and underground organs of the other species), and Bicyclogermacrene, δ-Cadinene and α-Cadinol (observed mainly in the EOs from the aerial organs of all four species).

The high number of compounds identified reflects the diversity of the chemical composition among the species. Even so, some compounds were unique to a given species (see Table 2): *Aldama bakeriana* (7 unique compounds), *A. discolor* (15), *A. grandiflora* (10) and *A. squalida* (6).

Multivariate analyses based on the occurrence of compounds in the oil revealed high similarity among the EOs from aerial and underground organs. Both cluster analysis (Figure 4) and the principal coordinate analysis (PCoA) (Figure 5) indicated two main groups: one group formed by EOs from leaves and aerial stems and a second group consisting of EOs from underground organs.

Although the chemical composition of the EOs was more similar among organs from different species, it was observed that into these two main groups, the EOs from a given species appeared as a cluster, revealing the chemical identity of each species. *Aldama bakeriana* and *A. discolor* were the species that presented the highest consistency in the group, while *A. grandiflora* and *A. squalida* were more similar based on the chemical composition of their EOs (Figure 4B).

4. Discussion

Four of the secretory structures described for the Asteraceae family (Castro et al., 1997) were found in the species analyzed: hydathodes, trichomes, canals and cavities. These findings are consistent with previous studies on *Aldama* (Bombo et al., 2012; Oliveira et al., 2013a; Bombo et al., 2014; Silva et al., 2014) and confirm the constancy of these structures in the group. Since they are consistent and widely distributed in plants and vary both morphologically and anatomically, these structures are usually identified as characters of diagnostic value for different species and genera (Solereder, 1908; Metcalfe and Chalk, 1950; Kelsey, 1984; Castroet al., 1997; Fahn, 2000). In this study, each species can therefore be identified based on the distribution of these structures, along with the chemical data generated.

Glandular trichomes have been widely studied in Asteraceae family representatives, and in particular the capitate glandular trichome, because it is associated with the production of terpenoids, flavonoids and other resinous metabolites (Duke et al., 2000; Fahn, 2000; Göpfert et al., 2009; Aschenbrenner et al., 2015; Bombo et al., 2015). Trichomes can change the optical properties of the leaf surface and may help conserve heat and/or moisture (Wagner, 1991), as well as protecting the plant against solar radiation. Histochemical screening performed here, enabled us to detect lipophilic substances and, more specifically, the presence of essential oils in this trichome.

Regarding the linear glandular trichome, morphological and anatomical studies are still lacking in the literature and only a few authors have highlighted its presence in Asteraceae family representatives (Castro et al., 1997; Aschenbrenner et al., 2013; Oliveira et al., 2013a). In the species analyzed herein, and also in *Aldama arenaria*, *A. robusta*, *A. kunthiana* and *A. tenuifolia* (Oliveira et al., 2013a; Silva et al., 2014), these trichomes accumulate phenolic compounds as well as lipophilic and mucilaginous substances. Castro et al. (1997) have already reported the occurrence of this type of trichome in the *Aldama* (= *Viguiera*) genus, but did not comment on the nature of its secretion. In *Helianthus annuus*, a species taxonomically very close to the *Aldama* genus, Aschenbrenner et al. (2013) and Spring et al. (1992) reported the accumulation of terpenes in linear trichomes.

Canals and cavities are common structures described extensively in the Asteraceae family (Solereder, 1908; Metcalfe and Chalk, 1950; Ramayya, 1962; Cury and Appezzato-da-Glória, 2008), including the *Aldama* genus (Bombo et al., 2012; Oliveira et al., 2013a; Bombo et al., 2014; Silva et al., 2014). These secretory structures are often associated with veins, mainly near the phloem, in several species (Bartoli et al., 2011; Plos et al., 2011; Bombo et al., 2012; Oliveira et al., 2013a), and in the conducting tissue they could play a part in protecting against herbivores and pathogens in the vascular system. For the species analyzed herein, canals and cavities were primarily responsible for producing essential oils, since EO yield was higher in the underground system organs, which contained higher amounts of EO and larger secretory

structures. The leaves were found to secrete both lipophilic substances and mucilage, and in the underground organs of *A. bakeriana*, alkaloid production was also detected in the secretory structures, and the mixed nature of the secretions in these organs may have contributed to the lower essential oil yield found.

Histochemical screening in species that have secretory structures can be useful in providing a preliminary indication of the location and type of secretion (Ascensao and Pais, 1988). The lipophilic substances characterized herein by the presence of essential oils were identified in capitate glandular trichomes, secretory internal structures, and also accumulated as droplets in the mesophyll. There was no variation in the classes of compounds detected in the species analyzed, which demonstrates how consistent these substances are in representatives of the group.

For the species analyzed herein, EO yields and chemical composition varied from one species to another and from one organ to another in a given species. Both EO yield and chemical composition can be influenced by several factors, such as genotype, developmental stage of the plant and the environmental conditions to which individuals are exposed (Marotti et al., 1994; Castro et al., 2006; Deschamps et al., 2008). They can vary significantly even among populations at different locations (Morais, 2009) and the variation in yield and composition observed herein was expected. However, correct chemical characterization of a species must be based on the pattern of occurrence of the components in essential oils from different plant individuals and organs (Erdtman, 1973) in order to take account of all the variation induced by these factors. With the aim of chemically characterizing each species, we sampled populations at least 30 km apart in order to cover variations in chemical composition in response to different locations, and we can assume that the chemical profile presented here for each species in fact characterize them.

Despite variations in chemical composition among the EOs from the four species studied, 13 compounds were common to all of them. Several constituents identified in this study have already been reported for other *Aldama* species (Bombo et al., 2012; 2014), showing that the genus is chemically consistent. However, some compounds were unique to a given species. The set of compounds, whether unique or common to the group, constitutes the chemical profile for each species and can be used for identification purposes.

Statistical analyses identified close similarity in the chemical composition of EOs from the same organs in different species. It is known that the developmental stage of the organ and even the type of organ can determine the composition of the EO (Figueiredo et al. 2008). The close similarity found among EOs from the same organs in different species indicates that the chemical composition of the EOs in these species is closely related to the function and/or positioning of the organ in the plant body. Even where the EOs from different species were similar, analysis also indicated that each species can be grouped according EO chemical

composition, to form a chemically consistent group based on the aerial or underground EOs from each species.

Several compounds reported herein have been proven to be biologically or pharmacologically active: Linalool, Limonene, Myrcene, α- and β-Pinene and Terpinen-4-ol affect the cardiovascular system (Santoset al., 2011), and bactericidal activity has been reported in studies on other Asteraceae species (Leite et al., 2007; Canales et al., 2008). In addition, dozen of volatiles are emitted by herbivore-damaged plants (Maffei et al. 2010). As well as providing important chemotaxonomic data for species delimitation (Harborne and Turner, 1984; Alvarengaet al., 2001; Emerenciano et al., 2001; Alvarenga et al., 2005; Bombo et al., 2012), identifying chemical compounds and synthesis sites in the plant body can also assist in surveying possible bioactive compounds, indicating the best organs for extraction and how the target constituents can best be exploited.

5. Conclusions

The secretory structures of the *Aldama* species analyzed herein are typical for this genus. Histochemical tests indicate that essential oils are produced by capitate glandular trichomes, but mainly by the canals and cavities found in the underground organs, which exhibited the higher yield values, especially in *A. grandiflora* and *A. squalida*. The chemical composition of the four species, described herein for the first time, reveals several compounds of proven biological activity, confirming the potential usefulness of *Aldama* species.

6. References

Adams, R.P., 2007. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry. Allured Publishing Corporation, Illinois.

Alvarenga, S.A.V., Ferreira, M.J.P., Rodrigues, G.V., Emerenciano, V.O., 2005. A general survey and some taxonomic implications of diterpenes in the Asteraceae. Bot. J. Linn. Soc.147, 291-308.

Alvarenga, S.A.V., Ferreira, M.J.P., Emerenciano, V.O., Cabrol-Bass, D., 2001. Chemosystematic studies of natural compounds isolated from Asteraceae: characterization of tribes by principal component analysis. Chemometr. Intell. Lab.56, 27-37.

Ambrosio, S.R., Schoor, K., Da Costa, F.B., 2004. Terpenoids of *Viguiera arenaria* (Asteraceae). Biochem. Syst. Ecol. 32, 221-224.

Appezzato-da-Glória, B., Hayashi, A.H., Cury, G., Soares, M.K.M., Rocha, R., 2008. Occurrence of secretory structures in underground systems of seven Asteraceae species. Bot. J. Linn. Soc. 157, 789-796.

Appezzato-da-Glória, B., Bombo, A.B., Oliveira, A.S.S., Ruiz, A.L.T.G., Carvalho, J.E., Rehder, V.L.G. 2012. Chemical composition, byosinthesis sites and evaluation of *in vitro* antiproliferative activity of essential oils of *Aldama filifolia* (Asteraceae**)**. Annals of 43rd International Symposium on Essential Oils 2012. Lisboa, Portugal.

Arakawa, N.S., Schorr, K., Ambrósio, S.R., Merfort, I., Da Costa, F.B., 2008. Further sesquiterpene lactones from *Viguiera robusta* and the potential anti-inflammatory activity of a heliangolide: inhibition of human neutrophil elastase release. Z. Naturforsch. C. 63, 533-538.

Ascensao, L., Pais, M., 1988. Ultrastructure and histochemistry of secretory ducts in *Artemisia campestris* ssp *maritima* (Compositae). Nord. J. Bot. 8, 283-292.

Aschenbrenner, A.K., Amrehn, E., Bechtel, L., Spring, O., 2015. Trichome differentiation on leaf primordia of *Helianthus annuus* (Asteraceae): morphology, gene expression and metabolite profile. Planta 241, 837-846.

Aschenbrenner, A. K., Horakh, S., Spring, O., 2013. Linear glandular trichomes of *Helianthus* (Asteraceae): morphology, localization, metabolite activity and occurrence. AoB Plants 5, 1-9.

Bartoli, A., Galati, B., Tortosa, R., 2011. Anatomical studies of the secretory structures: Glandular trichomes and ducts, in *Grindelia pulchella* Dunal (Astereae, Asteraceae). Flora 206, 1063-1068.

Bombo, A.B., Appezzato-da-Glória, B., Aschenbrenner, A.K., Spring, O., 2016. Capitate glandular trichomes in *Aldama discolor* (Heliantheae - Asteraceae): morphology, metabolite profile and sesquiterpene biosynthesis. Plant Biol. 18, 455-62.

Bombo, A.B., Oliveira, T.S., Oliveira, A.S.S., Rehder, V.L.G., Appezzato-da-Glória, B., 2014. Anatomy and essential oil composition of the underground systems of three species of *Aldama* La Llave (Asteraceae). J. Torrey Bot. Soc.141, 115-125.

Bombo, A.B., Oliveira, T.S., Oliveira, A.S.S., Rehder, V.L.G., Magenta, M.A.G., Appezzato-da-Glória, B., 2012. Anatomy and essential oils from aerial organs in three species of *Aldama* (Asteraceae-Heliantheae) that have a difficult delimitation. Aust. J. Bot.60, 632-642.

Cain, A. J., 1947. The use of nile blue in the examination of lipoids. Q. J. Microsc. Sci. 88,383-392.

Canales, M., Hernández, T., Rodríguez-Monroy, M.A., Jiménez-Estrada, M., Flores, C.M., Hernández, L.B., Gijón, I.C., Quiroz, S., García, A.M., Ávila, G., 2008.Antimicrobial activity of the extracts and essential oil of *Viguiera dentata*. Pharma. Biol. 46, 719-723.

Carvalho, T.C., Simão, M.R., Ambrósio, S.R., Furtado, N.A., Veneziani, R.C., Heleno, V.C., Da Costa, F.B., Gomes, B.P., Souza, M.G., Reis, E.B., Martins, C.H., 2011. Antimicrobial activity of diterpenes from *Viguiera arenaria* against endodontic bacteria. Molecules 16, 543-551.

Castro, M.D.M., Leitão-Filho, H.D.F., Monteiro, W.R., 1997. Utilização de estruturas secretoras na identificação dos gêneros de Asteraceae de uma vegetação de cerrado. Braz. J. Bot. 20, 163-174.

Castro, D.P., Cardoso, M.G., Moraes, J.C., Santos, N.M., Baliza, D.P., 2006. Não-preferência de *Spodoptera frugiperda* (Lepidoptera: Noctuidae) por óleos essenciais de *Achillea millefolium* L. e *Thymus vulgaris* L. Rev. Bras. Plantas Med.8, 27-32.

Clark, G., 1973. Staining procedures. 3rd ed. The Williams & Wilkins: Baltimore.

Cury, G., Appezzato-da-Glória, B., 2009. Internal secretory spaces in thickened underground systems of Asteraceae species. Aust. J. Bot. 57, 229-239.

David, R., Carde, J.P., 1964. Coloration différentielle des inclusions lipidique et terpeniques des pseudophylles du Pin maritime au moyen du reactif Nadi. CR. Acad. Sci. 258, 1338-1340.

Deschamps, C., Zanatta, J.L., Bizzo, H.R. , Oliveira, M.C. , Roswalka, L.C., 2008. Seasonal evaluation of essential oil yield of mint species. Cienc. Agrotec. 32, 725-730.

Duke, S.O., Canel, C., Rimando, A.M., Tellez, M.R., Duke, M.V., Paul, R.N., 2000. Current and Potential Exploitation of Plant Glandular Trichome Productivity, in: Hallahan, D.L., Gray, J.C. (Eds.), Plant Trichomes. Academic Press, San Diego, pp. 121-152.

Erdtman, H., 1973. Some aspects on chemotaxonomy**.** Academic Press, London.

Emerenciano, V., Militão, J.S.L.T., Campos, C.C., Romoff, P. Kaplan, M.A.C., Zambon, M., Brant, A.J.C., 2001. Flavonoids as chemotaxonomic markers for Asteraceae. Biochem. Syst. Ecol.29, 947-957.

Fahn, A., 2000. Structure and function of secretory cells. Adv. Bot. Res. 31, 37-75.

Figueiredo, A.C., Cristina, A., Barroso, J.G., Pedro, L.G., Scheffer, J.J., 2008. Factors affecting secondary metabolite production in plants: volatile components and essential oils. Flavour Frag. J. 23, 213-226.

Furr, M., Mahlberg, P.G., 1981. Histochemical analyses of lacticifers and glandular trichomes in *Cannabis sativa*. J. Nat. Prod.44, 153-159.

Göpfert, J.C., Macnevin, G., Ro, D.K., Spring, O., 2009. Identification, functional characterization and developmental regulation of sesquiterpene synthases from sunflower capitate glandular trichomes. BMC Plant Biol. 9, 86.

Gotelli, N.J., Ellison, A.M., 2011. Princípios de estatística em ecologia, first ed. Artmed, Porto Alegre.

Gregory, M., Baas, P., 1989. A survey of mucilage cells in vegetative organs of the dicotyledons. Israel J. Bot. 38, 125-174.

Harborne, J.B., Turner, B.L., 1984. Plant Chemosystematics. Academic Press, London.

Horridge, G.A., Tamm, S.L., 1969. Critical point drying for scanning electron microscopy study of ciliary motion. Science 163, 817-818.

Hughes, J., Mccully, M.E., 1975. The use of an optical brightener in the study of plant structure. Stain Techn. 50, 319-329.

Jensen, W., 1962. Botanical histochemistry, principles and practice. W.H. Freeman, San Francisco.

Johansen, D., 1940. Plant microtechnique. McGraw-Hill Book Co. Inc., New York.

Karnovsky, M.J., 1965. A Formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol.27, 137-138.

Kelsey, R., 1984. Glandular trichomes - a helpful taxonomic character for *Artemisia nova* (black sagebrush). J. Range Manage.37, 370-372.

Kirk Jr., P.W., 1970. Neutral red as a lipid fluorochrome. Stain Techn. 45, 1-4.

Leite, A.M., Lima, E.O., Souza, E.L., Diniz, M.F.F.M., Trajano, V.N., Medeiros, I.A., 2007. Inhibitory effect of β-pinene, α-pinene and eugenol on the growth of potential infectious endocarditis causing Gram-positive bacteria. Braz. J. Pharm. Sci. 43, 121-126.

Maffei, M.E., 2010. Sites of synthesis, biochemistry and functional role of plant volatiles. South Afr. J. Bot. 76, 612-631.

Magenta, M.A.G., Pirani, J.R., 2014. Novidades taxonômicas em *Aldama* (Asteraceae-Heliantheae). Rodriguésia65, 175-192.

Marquina, S., Maldonado, N., Garduño-Ramírez, M.L., Aranda, E., Villarreal, M.L., Navarro, V., Bye, R., Delgado, G., Alvarez, L., 2001. Bioactive oleanolic acid saponins and other constituents from the roots of *Viguiera decurrens*. Pytochemistry 56, 93-97.

Marotti, M., Piccaglia, R., Giovanelli, E., Deans, S.G., Eaglesham, E., 1994. Effects of planting time and mineral fertilization on peppermint (*Menthapiperita* L.) essential oil composition and its biological activity. Flavour Frag. J. 9, 125-129.

Morais, L.A.S.D., 2009. Influência dos fatores abióticos na composição química dos óleos essenciais. Hort. Bras. 27, S4050- S4063.

Mccaskill, D., Croteau, R., 1999. Strategies for bioengineering the development and metabolism of glandular tissues in plants. Nature Biotechnol. 17, 31-36.

Mcmanus, J., 1948. Histological and histochemical uses of periodic acid. Stain Techn. 23, 99-108.

Metcalfe, C. R., Chalk, L., 1950. Anatomy of the dicotyledons. Clarendon Press, Oxford.

Nicolete, R., Arakawa, N.S., Rius, C., Nomizo, A., Jose, P.J., Da Costa, F.B., Sanz, M.J., Faccioli, L.H., 2009. Budlein A from *Viguiera robusta* inhibits leukocyte-endothelial cell interactions, adhesion molecule expression and inflammatory mediators release. Phytomedicine16, 904-915.

Oliveira, A.S.S., Rehder, V.L.G., Ruiz, A.L.T.G., Sousa, V.H.S., Carvalho, J.E., Almeida, T.S.O., Appezzato-Da-Glória, B., Imamura, P.M., 2013a. Atividade antiproliferativa *in vitro* do óleo essencial e frações de *Aldama arenaria* Baker (Sin.: *Viguiera arenaria*). 36ª Reunião Anual da Sociedade Brasileira de Química. Águas de Lindóia.

Oliveira, T.S.D., Bombo, A.B., Appezzato-da-Glória, B., 2013b. Anatomy of vegetative organs with an emphasis on the secretory structures of two species of *Aldama* (Asteraceae – Heliantheae). Botany91, 335-342.

Plos, A., Sancho, G., Iharlegui, L., 2011. Secretory structures of leaves of *Ophryosporus* Meyen (Asteraceae, Eupatorieae), a genus with medicinal properties. J. Torrey Bot. Soc.138, 391-399.

Porto, T.S., Furtado, N.A.J.C., Heleno, V.C.G., Martins, C.H.G., Da Costa, F.B., Severiano, M.E., Silva, A.N., Veneziani, R.C.S., Ambrósio, S.R., 2009. Antimicrobial ent-pimarane diterpenes from *Viguiera arenaria* against Gram-positive bactéria. Fitoterapia 80, 432-436.

Ramayya, N., 1962. Studies on the trichomes of some Compositae II. Phylogeny and Classification. Bull. Bot. Surv. India4, 189-192.

Rehder, V.L.G., Oliveira, A.S.S., Bombo, A.B., Ruiz, A.L.T.G., Carvalho, J.E., Appezzato-da-Glória, B., 2012. Evaluation of chemical composition and in vitro antiproliferative activity of essential oils of different parts of *Aldama linearifolia* Chodat. 43rd International Symposium on Essential Oils. Lisbon, Portugal.

Sakai, W., 1973. Simple method for differential staining of paraffin embedded plant material using toluidine blue O. Stain Techn. 48, 247-249.

Santos, M.R.V., Moreira, F.V., Fraga, B.P., Souza, D.P., Bonjardim, L.R., Quintans-Junior, L.J., 2011. Cardiovascular effects of monoterpenes: a review. Rev. Bras. Farmacog.21, 764-771.

Schilling, E.E., Da Costa, F.B., Lopes, N.P., Heise, P.J., 2000. Brazilian species of *Viguiera* (Asteraceae) exhibit low levels of its sequence variation. Edinburgh J. Bot. 57,: 323–332.

Sheperd, G.J., 2010. Fitopac 2.1. Manual do usuário. Departamento de Botânica, Unicamp. http://pedroeisenlohr.webnode.com.br/fitopac/ (accessed 28.04.16)

Silva, E. M. S. D., Hayashi, A. H., Appezzato-da-Glória, B., 2014. Anatomy of vegetative organs in *Aldama tenuifolia* and *A. kunthiana* (Asteraceae: Heliantheae). Braz. J. Bot.37, 505-517.

Sneath, P.H.A., Sokal, R.R., 1973**.** Numerical Taxonomy. Freeman, San Francisco.

Solereder, H., 1908. Systematic anatomy of the dicotyledons: A handbook for laboratories of pure and applied botany. Clarendon Press, Oxford.

Spring, O., Zipper, R., Conrad, J., Vogler, B., Klaiber, I., Da Costa, F.B., 2003. Sesquiterpene lactones from glandular trichomes of *Viguiera radula* (Heliantheae; Asteraceae). Phytochemistry 62, 1185-1189.

Spring, O., Rodon, U., Macias, F., 1992. Sesquiterpenes from noncapitate glandular trichomes of *Helianthus annuus*. Phytochemistry31, 1541-1544.

Statsoft Inc., 2007. STATISTICA for windows. Statsoft Inc. 2300, East 14th Street, Tulsa, 91, 339-341.

Strasburger, E., 1913. Handbook of practical botany. George Allen & Company Ltda., London.

Valério, D.A.R., Cunha, T.M., Arakawa, N.S., Lemos, H.P., Da Costa, F.B., Parada, C.A., Ferreira, S.H., Cunha, F.Q., Verri, W.A., 2007. Anti-inflammatory and analgesic effects of the sesquiterpene lactone budlein A in mice: inhibition of cytokine production-dependent mechanism. Eur. J. Pharmacol. 562, 155-163.

Wagner, G., 1991. Secreting glandular trichomes - more than just hairs. Plant Physiol.96, 675-679.

Table 1. Fresh matter (grams g) used for essential oil extraction from leaves, aerial/underground stems and roots of *Aldama* species.

Species	***A. bakeriana***			***A. discolor***			***A. grandiflora***			***A. squalida***	
Population	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**
Leaves	40.15	62.55	63.12	217.86	295.80	381.76	103.03	109.36	69.29	222.47	361.05
Aerial stems	69.84	97.83	104.55	128.65	152.90	229.19	120.96	79.46	58.86	161.72	449.22
Underground stems	62.98	147.36	47.88	77.45	171.91	50.28	122.22	41.15	41.27	161.05	184.33
Roots	169.44	185.59	39.23	109.17	269.91	158.96	166.45	254.54	174.16	375.94	946.22

Table 2. Chemical composition and yield (% w/w) of the essential oils from leaves (L), stems (S), roots (R) and underground stems (UndS) of *Aldama bakeriana*, *A. discolor*, *A. grandiflora* and *A. squalida.* AI calc = calculated arithmetic index; AI lit = arithmetic index found in the literature. Values in bold type correspond to the major compounds in the EOs from at least one organ of the species. Values = mean ± standard deviation.

Compound	AI calc	AI lit	*Aldama bakeriana*				*Aldama discolor*				*Aldama grandiflora*				*Aldama squalida*			
			L	S	R	UndS	L	S	R	UndS	L	S	R	UndS	L	S	R	UndS
Yield (% w/w)			0,01 ± 0,01	0,01 ± 0,01	0,05 ± 0,02	0,03 ± 0,02	0,08 ± 0,02	0,02 ± 0,01	0,07 ± 0,02	0,05 ± 0,05	0,18 ± 0,07	0,02 ± 0,01	0,31 ± 0,11	0,29 ± 0,16	0,06 ± 0,06	0,05 ± 0,05	0,10 ± 0,03	0,22 ± 0,07
α-Thujene	925	924							2.04 ± 1.87								0.92 ± 1.30	0.43 ± 0.61
α-Pinene	933	932					6.04 ± 9.1	2.29 ± 3.84	3.64 ± 5.2	0.38 ± 0.25	**4.29 ± 7.20**	**0.95 ± 1.35**	**65.52 ± 16.69**	**58 ± 7.18**	**3.93 ± 4.77**	**12.37 ± 17.49**	**19.66 ± 16.87**	**12.23 ± 11.31**
Camphene	947	946									0.78 ± 1.35	0.17 ± 0.29	2.98 ± 0.94	1.41 ± 0.91			0.53 ± 0.75	
Sabibene	972	969					0.93 ± 1.1	0.25 ± 0.43			1.72 ± 2.98	1.27 ± 2.21	1.57 ± 0.08	0.74 ± 0.68	1.79 ± 1.36	4.4 ± 1.17	0.84 ± 1.18	
β-Pinene	974	974	0.11 ± 0.18				1.53 ± 1.5	0.43 ± 0.64	4.46 ± 6.45	0.6 ± 0.84	1.7 ± 2.95	1.32 ± 2.29	11.94 ± 17.89	9.7 ± 16.14	1.07 ± 0.87	3.52 ± 4.97	5.38 ± 0.65	0.23 ± 0.33
β-Myrcene	990	988	0.14 ± 0.23	3.74 ± 5.96			0.42 ± 0.2	0.62 ± 0.58			1.17 ± 0.91	0.98 ± 0.11			0.23 ± 0.32	1.39 ± 1.97	0.35 ± 0.49	0.4 ± 0.56
3-Carene	1010	1008							5.06 ± 4.79	0.08 ± 0.10			0.56 ± 0.60		**0.47 ± 0.07**		**15.88 ± 4.52**	**2.19 ± 2.02**
o-Cymene	1024	1022							1.33 ± 1.82	0.57 ± 0.45								
D-Limoneno	1027	1024	0.79 ± 0.74	1.07 ± 1.45			2.92 ± 2.7	1.8 ± 2.81	0.91 ± 0.78				1.75 ± 1.10	1.38 ± 1.39	1.07 ± 0.25	3.33 ± 2.69	8.06 ± 4.58	0.24 ± 0.33
β-Phellandrene	1030	1025									5.78 ± 10.01	4.02 ± 6.96						
β-cis-Ocimene	1037	1032	0.29 ± 0.50															
Phenyl ethanol	1044	1036	0.82 ± 1.06															
β-trans-Ocimene	1046	1044	2.34 ± 1.72	1.09 ± 1.31							2.64 ± 1.48	0.14 ± 0.24			0.77 ± 1.08			
γ-Terpinene	1057	1054														0.59 ± 0.83		
Terpinolene	1088	1086									0.44 ± 0.76	0.31 ± 0.32						
α-Pinene oxide	1100	1099					0.28 ± 0.5											
Linalol	1102	1095		0.72 ± 0.90														
L-4-Terpineol	1177	1174						1 ± 0.37	1.61 ± 1.87	4.27 ± 6.03		1.75 ± 3.03			0.52 ± 0.73	6.3 ± 5.27		
L-Verbenone	1210	-					0.18 ± 0.3											
Bornyl acetate	1285	1287							0.73 ± 0.93		0.61 ± 1.06	1.77 ± 1.42	1.94 ± 0.51					
Anetol	1287	1282		0.53 ± 0.91														
Silfinene	1342	1347											0.69 ± 0.60					
α-Cubebene	1349	1345																1.15 ± 0.98
Copaene	1375	1374							1.07 ± 1.19	0.42 ± 0.43	0.64 ± 0.25	1 ± 0.37			0.36 ± 0.51	1.17 ± 1.65	0.79 ± 1.12	
α-Isocomene	1386	1387									1.87 ± 1.62	0.53 ± 0.92						
β-Cubebene	1387	1387	1.16 ± 1.0													0.44 ± 0.62	0.36 ± 0.51	0.42 ± 0.59
β-Elemene	1392	-					0.22 ± 0.3											
Cyperene	1396	1398			6.43 ± 1.14	1.94 ± 1.96			**18.11 ± 0.94**	**6.43 ± 0.35**			0.42 ± 0.72				3.76 ± 5.31	1.62 ± 2.28
β-Isocomene	1405	1408											0.7 ± 0.60	0.21 ± 0.36				
t-Caryophyllene	1418	1417	14.62 ± 4.97	6.84 ± 5.10			1.72 ± 0.1	1.94 ± 0.35		1.71 ± 0.15	0.86 ± 0.79	1.17 ± 0.48		0.34 ± 0.59	1.01 ± 0.69	0.5 ± 0.70	0.52 ± 0.73	1.45 ± 1.10
6,9-Guaiadiene	1443	1442											0.58 ± 1.00	0.3 ± 0.52				
α-Humulene	1453	1452	3.58 ± 1.26	0.73 ± 0.76			2.3 ± 1.2	1.36 ± 0.24			0.53 ± 0.56	0.21 ± 0.18			0.34 ± 0.48			
τ-Muurolene	1477	1478							1.07 ± 0.93	0.84 ± 1.18					0.39 ± 0.55			
Germacrene D	1481	1482	**41.91 ± 9.70**	**17.05 ± 4.10**			**2.88 ± 1.9**	**6.97 ± 2.28**	**2.49 ± 2.37**	**22.53 ± 14.92**	**19.15 ± 7.45**	**18.02 ± 9.79**	**4.84 ± 4.08**	**13.86 ± 10.05**	**11.99 ± 14.19**	**10.99 ± 2.99**	**12.85 ± 4.36**	**26.5 ± 26.68**
10s,11s-Himachala-3(12),4-diene	1483	-									1.13 ± 1.96				**5.51 ± 7.79**	**11.82 ± 16.72**	**4.48 ± 6.33**	**4.89 ± 6.91**
β-Selinene	1485	1489	0.17 ± 0.30						0.78 ± 0.29	0.17 ± 0.23								
Valencene	1494	1496	0.18 ± 0.30															
(Z,E)-α-Farnesene	1495	-			0.65 ± 0.60	0.78 ± 0.72												
Bicyclogermacrene	1495	1500	**14.28 ± 5.02**	**24.77 ± 16.38**			2.27 ± 0.8	4.43 ± 3.10		1.03 ± 1.45	**13.64 ± 3.19**	**18.9 ± 8.90**	**0.3 ± 0.51**	**2.73 ± 3.96**	**11.68 ± 11.91**	**4.76 ± 5.42**		**1.04 ± 1.47**
α-Muurolene	1500	1500									0.89 ± 0.81	0.27 ± 0.47						
α-Bulnesene	1505	1509	1.3 ± 1.42	0.93 ± 1.15														
β- Bisabolene	1508	1505				0.69 ± 0.60			0.64 ± 0.63	0.16 ± 0.21							2.23 ± 1.08	
γ-Cadinene	1513	1511									0.36 ± 0.62							
δ-Amorphene	1514	1513									0.4 ± 0.69							
Cubebol	1515	1514		0.69 ± 1.19			2.18 ± 0.9	0.76 ± 0.13			1.27 ± 0.52	0.89 ± 0.23			1.46 ± 0.51			
δ-Cadinene	1522	1522	1.85 ± 0.07	3.04 ± 0.84			0.83 ± 0.8	1.12 ± 0.27		1.62 ± 0.70	6.06 ± 3.93	3.53 ± 1.83	0.82 ± 1.41	0.65 ± 1.13	5.2 ± 2.33	3.62 ± 2.91	6.66 ± 9.42	7.84 ± 8.29
α-Calacorene	1542	1544													1.18 ± 1.66			
Elemol	1554	1548					2.59 ± 4.5											
Nerolidol	1565	1561		1.48 ± 1.33	0.76 ± 0.31	1.71 ± 1.53	0.6 ± 0.3											
Germacrene D-4-ol	1576	1574	5.09 ± 1.75	2.56 ± 4.43							**7.42 ± 7.10**	**6.24 ± 5.44**						
Spathulenol	1578	1577		1.66 ± 1.46	0.77 ± 0.24		6.22 ± 5.6	5.68 ± 4.17	0.58 ± 0.53	0.7 ± 0.33	0.73 ± 0.64	5 ± 1.62		0.52 ± 0.91	**20.14 ± 11.88**	**5.88 ± 3.37**	**0.64 ± 0.90**	**0.41 ± 0.58**
Globulol	1584	1590		1.19 ± 1.37							0.73 ± 0.28	1.3 ± 0.40			2.62 ± 0.01			
Caryophyllene oxide	1585	1582					4.86 ± 3.5	1.54 ± 0.33										
Viridiflorol	1592	-		0.85 ± 0.75			4.78 ± 5.5	2.07 ± 1.88			0.48 ± 0.42	1.28 ± 0.52		0.37 ± 0.64	4.31 ± 6.09			
Humulen-1,2-epoxide	1610	-		0.41 ± 0.71			2.23 ± 2.10	0.82 ± 0.48										
Eudesmol<10-epig>	1618	-	0.29 ± 0.50								0.48 ± 0.83	0.72 ± 1.25	0.49 ± 0.85	0.54 ± 0.94				
Cubenol <1 epi>	1628	-					0.56 ± 1.00	0.38 ± 0.19							1.72 ± 1.29			
τ-Eudesmol	1633	1630					0.61 ± 1.10	0.17 ± 0.29										
Elemol	1639	-		0.74 ± 1.28			0.95 ± 0.90	1.31 ± 1.03							0.61 ± 0.86			
α- Murulol	1642	1644	2.39 ± 0.74	4.14 ± 5.06							**7.74 ± 4.59**	**4.57 ± 2.64**			3.18 ± 0.62			
(+)-Epi-bicycle sesquiphellandrene	1642	-														2.76 ± 3.90		
τ-cadinol	1644	-	0.51 ± 0.48	0.63 ± 1.09			2.62 ± 0.90	1.89 ± 0.36										
Di-epi-α-cedrene-(I)	1650	-					2.86 ± 3.80	1 ± 0.90										
α-Eudesmol	1653	1652					0.63 ± 1.1				0.57 ± 0.98							
α-Cadinol	1654	1652	2.07 ± 1.68	6.26 ± 8.91			2.62 ± 2.80	1.81 ± 1.62			**11.74 ± 8.45**	**6.2 ± 3.62**			6.16 ± 2.08	0.9 ± 1.27		
α-Eudesmol	1655	1652					0.63 1.1											
Intermedeol <neo>	1662	-						6.01 ± 10.01										
Intermedeol	1667	-					**16.97 ± 29.40**											
Cycloisolongifole, 8-hydroxy-, endo-	1687	-					0.74 ± 0.90	1.02 ± 0.42										
Kaurene	2032	-						0.95 ± 0.36	2.14 ± 0.98	2.41 ± 0.93								0.78 ± 0.23
Kaur-16-ene	2033	2042														1.25 ± 1.77		
Manool	2055	2056					0.65 ± 1.1	0.29 ± 0.28	13.22 ± 12.53	8.45 ± 6.73								
8-β-podocarpan-8-ol	---	-						0.5 ± 0.65										
Pimaral	---	-						3.26 ± 1.33					1.62 ± 1.91	3.62 ± 2.24		**12.79 ± 8.13**	**3.37 ± 0.34**	**7.6 ± 10.74**
ent-8(14)-pimaridien-3 β-ol	---	-						6.12 ± 3.59	3.24 ± 2.76	4.51 ± 5.67		4.25 ± 6.01						
Lupenone	---	-					1.62 ± 2.8											
Kauran-16-ol	---	-								0.75 ± 1.06				0.4 ± 0.69				
16-β H-Kauran-16-ol	---	-												0.54 ± 0.94				
Kaurenoic acid methyl Ester	---	-																4.48 ± 3.22
MONOTERPENES			4.49	7.15	0	0	12.3	6.39	19.78	5.9	19.13	12.68	86.26	71.23	9.85	31.9	51.62	15.72
SESQUITERPENES			89.4	73.97	8.61	5.12	62.87	40.28	24.74	35.61	76.69	69.83	8.84	19.52	77.86	42.84	32.29	45.32
DITERPENES			0	0	0	0	2.27	11.12	18.6	16.12	0	4.25	1.62	4.56	0	14.04	3.37	12.86
Total of compounds identified			93.9	81.12	8.61	5.12	76.81	57.78	63.12	57.59	95.83	86.76	96.71	95.33	87.66	88.73	87.24	73.85

Table 3. Compounds common to all four *Aldama* species analyzed. (+) indicates the presence of the compound in the essential oil from the organ/species. L = leaves; S = aerial stems; R = roots; UndS = underground stem.

Compounds	*Aldama bakeriana*				*Aldama discolor*				*Aldama grandiflora*				*Aldama squalida*			
	L	**S**	**R**	**UndS**	**L**	**S**	**R**	**UndS**	**L**	**S**	**R**	**UndS**	**L**	**S**	**R**	**UndS**
β-Pinene	+				+	+	+	+	+	+	+	+	+	+	+	+
β-Myrcene	+	+			+	+			+	+			+	+	+	+
D-Limonene	+	+			+	+	+				+	+	+	+	+	+
Cyperene			+	+			+	+			+				+	+
t-Caryophyllene	+	+			+	+		+	+	+		+	+	+	+	+
α-Humulene	+	+			+	+			+	+			+			
Germacrene D	+	+			+	+	+	+	+	+	+	+	+	+	+	+
Bicyclogermacrene	+	+			+	+		+	+	+	+	+	+	+		+
Cubebol		+			+	+			+	+			+			
δ-Cadinene	+	+			+	+		+	+	+	+	+	+	+	+	+
Spathulenol		+	+		+	+	+	+	+	+		+	+	+	+	+
Viridiflorol		+			+	+			+	+		+	+			
α-Cadinol	+	+			+	+			+	+			+	+		

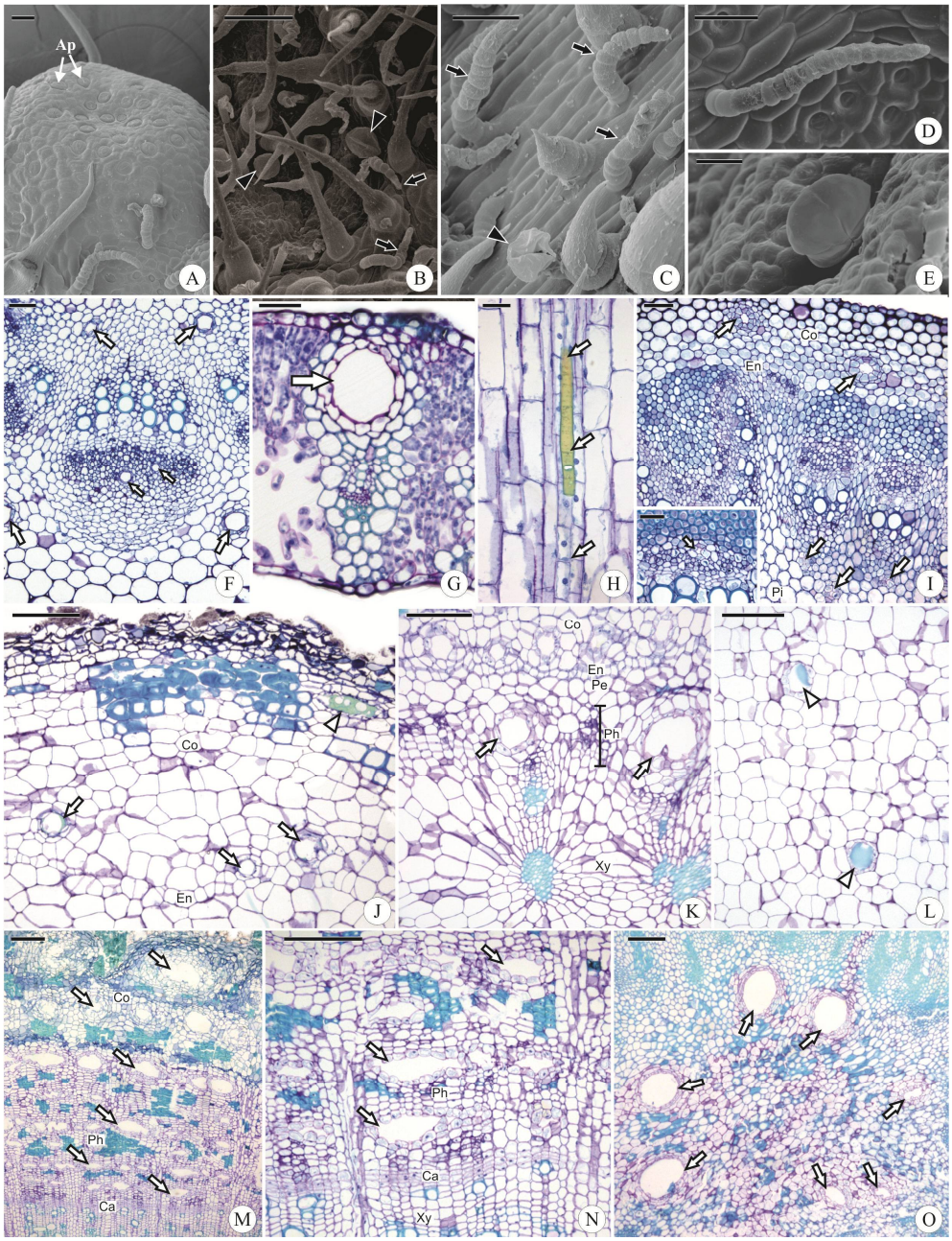

Figure 1. Scanning electron micrographs (A-E) and photomicrographs (F-O) from vegetative organs of *Aldama* species. A,C-F,J-K,O. *A. grandiflora*. B,I.*A. discolor*. G,L-N. *A. squalida*. H. *A. bakeriana*. Cross-sections (F-G, I-K, M-O) and longitudinal sections (H, L). A. Hydathode on leaf margin. B. Trichomes on the abaxial leaf epidermis. C. Trichomes on stem epidermis. D-E. Linear glandular (D) and capitate glandular (E) trichome. F-G. Canals in the fundamental parenchyma and phloem of the midrib (F) and in the sheath extensions of a lateral bundle (G). H. Secretory canal in aerial stem pith. I. Distribution of the canals in aerial stem. Canal observed in primary phloem (inset, arrow). J-L. Secretory structures in the roots. J. Cavity (*arrowhead*) and cortical canals. K. Canals in secondary phloem, arrows. I. Cavities in parenchymatic pith. M-O. Secretory canals in the underground stem. M. Distribution of the secretory canals. N. Canals in the secondary phloem. O. Canals in pith parenchyma. Black arrows in B and C: linear glandular trichomes; arrowheads: capitate glandular tricomes. Arrowheads in I and L: cavities; arrows in the other photos: secretory canals. Ap = aquiferous pore; Ca = cambium; Co = cortex; En = endodermis; Pe = pericycle; Ph = phloem; Xy = xylem. Scale bars: A-B,J,L = 100 µm; C-D,F-G,I = 50 µm; E = 20 µm; H and inset in I = 30 µm; K,N-O = 200 µm; L = 250 µm.

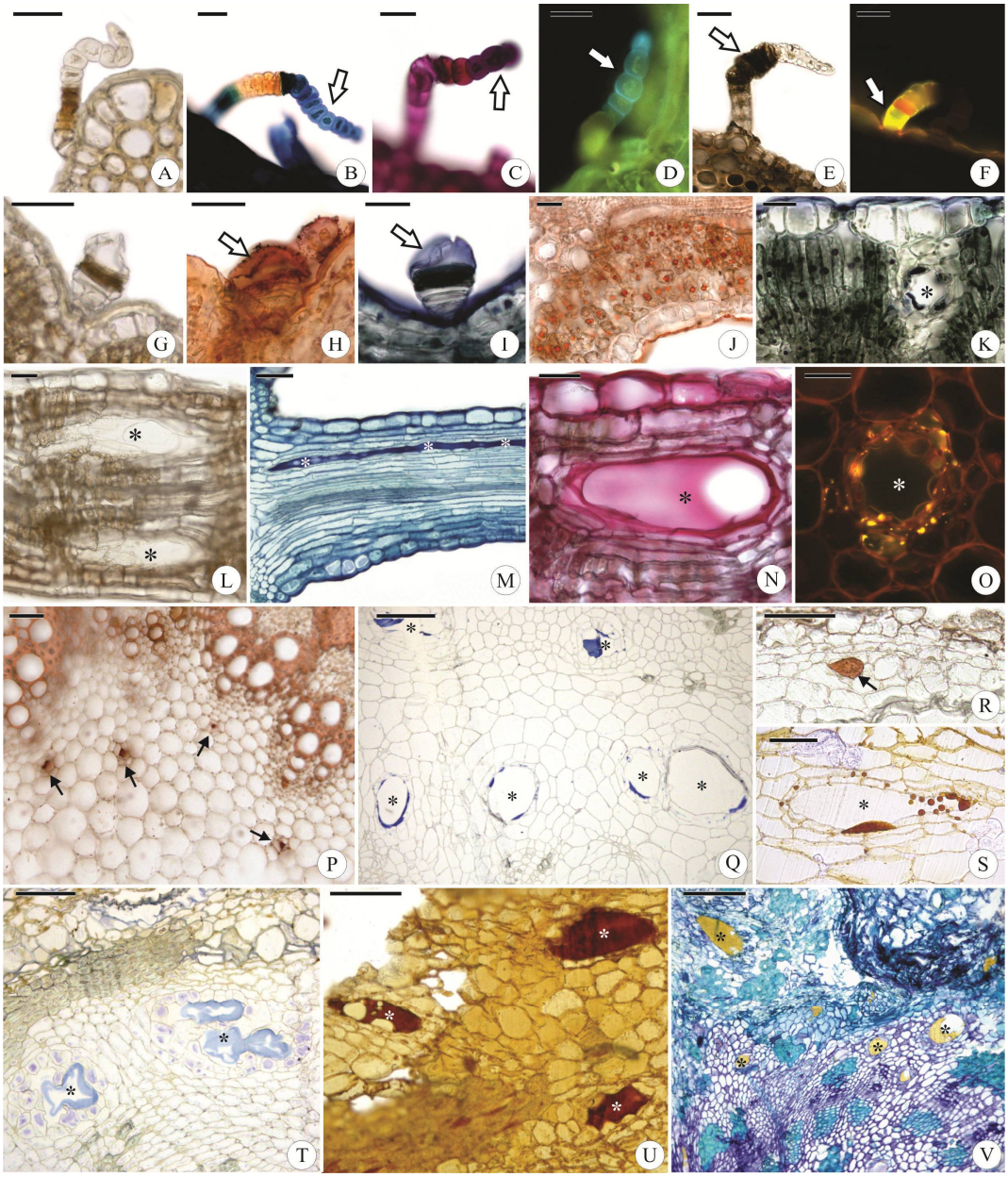

Figure 2. Histochemical screening in *Aldama* species. A-D,G-K,M,O,Q. *A. grandiflora*. E-F. *A. discolor*. L,N,V. *A. squalida*. P,R-U. *A. bakeriana*. A-F. Linear glandular trichome. G-I. Capitate glandular trichome. J-K. Droplets in the mesophyll under positive reaction to Sudan IV (J) and NADI reagent (K). L-S. Secretory canals in the leaves (L-O), stems (P, arrows), roots (Q,S), underground stem (T-U), and secretory cavity in the root (R, arrow). A,G,L. Non-stained sections. B-C,N. Pectic substances under positive reaction to methylene blue (B), PAS reagent (C) and ruthenium red (N). D. Apical cell walls stained with Calcofluor white. E. Phenolic compounds stained with ferric chloride reagent. F,O. Neutral red. H,J,P,R. Sudan IV. I,K,Q,T. NADI reagent. M. Nile blue sulfate. S,U. Wagner reagent. V. Methylene blue, highlighting yellowish substances with negative reaction to this stain. Arrows in B-F,H-I indicate positive reaction to the stain. * = lumina of the secretory canals. Scale bars: A,G,L,N,P-R = 50 µm; B-F,H-K,O,S = 30 µm; M,T-U = 100 µm; V = 200 µm.

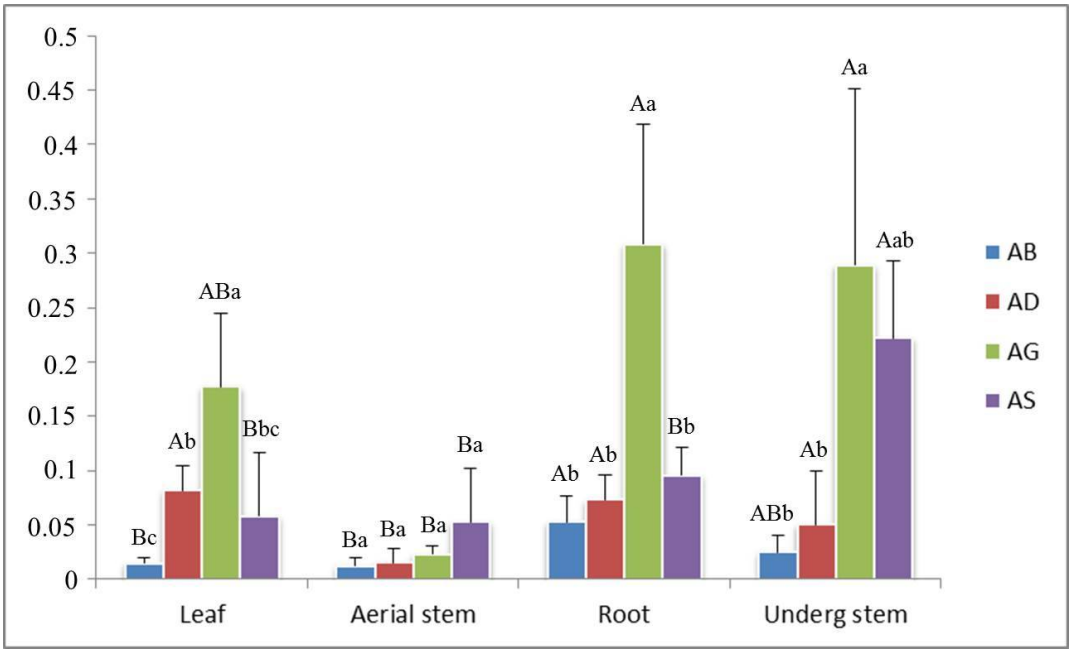

Figure 3. Essential oils yield (% w/w) in each species, for each organ analyzed. AB = *Aldama bakeriana*, AD = *A. discolor*, AG = *A. grandiflora*, AS = *A. squalida*. Capital letters compare EO yields from different organs of the same species; lowercase letters compare EO yields of the same organs from different species. Identical letters did not differ statistically.

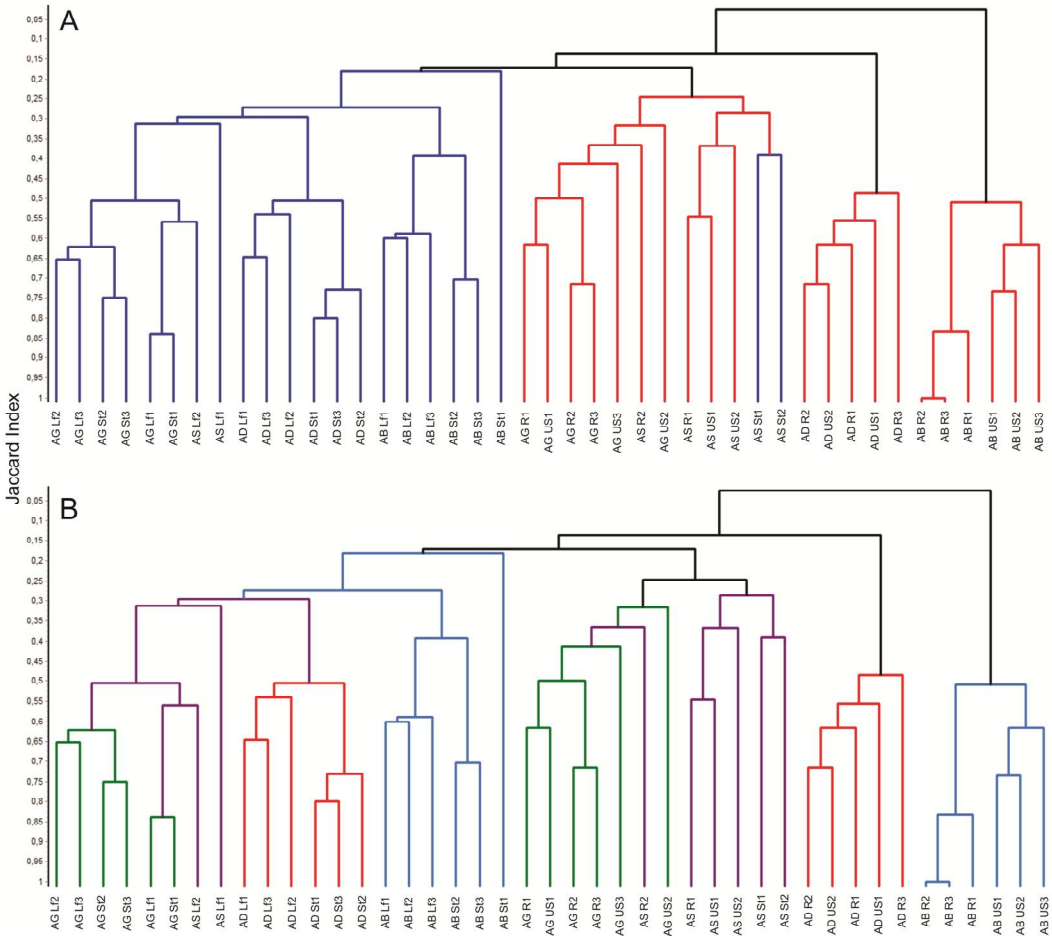

Figure 4. Cluster analyses of *Aldama* essential oils (presence or absence of compound) for each species, based on Jaccard's distance. Colors in A represent the organ position relative to soil level (aerial = blue; underground = red) and in B, different colors indicate different species (blue = *A. bakeriana* AB, red = *A. discolor* AD, green = *A. grandiflora* AG, purple = *A. squalida* AS). L = leaves, S = aerial stems, R = roots, US = underground stems. The numbers identify the samples analyzed.

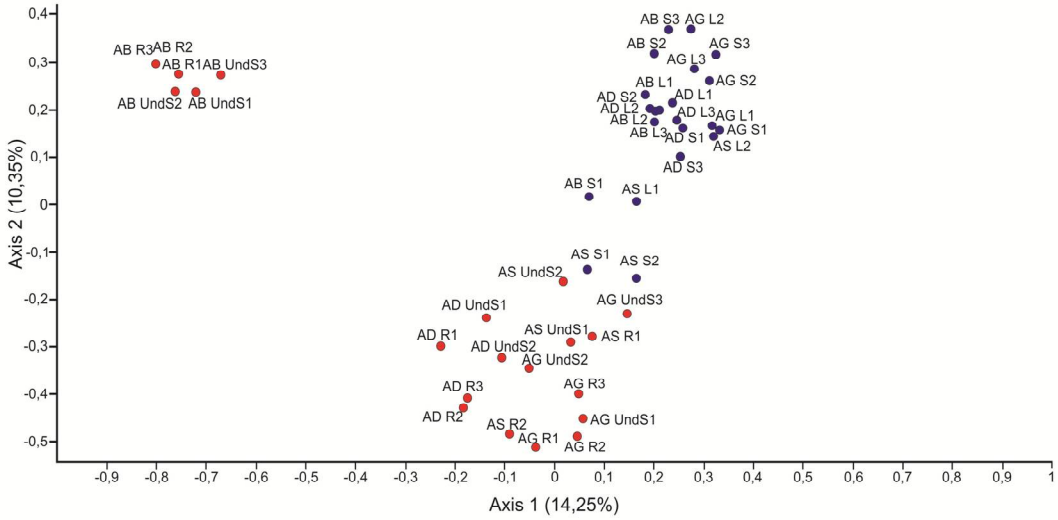

Figure 5. Principal coordinate analysis of *Aldama* essential oils (presence or absence of compound) for each species. Colors represent the organs position in relation to the soil level (aerial = blue; underground = red). AB = *A. bakeriana*; AD = *A.* discolor; AG = *A. grandiflora*; AS = *A. squalida* AS). L = leaves, S = aerial stems, R = roots, UndS = underground stems. The numbers identify the samples analyzed.

CAPÍTULO IV

Capitate glandular trichomes in *Aldama discolor* (Heliantheae – Asteraceae): morphology, metabolite profile and sesquiterpenes biosynthesis

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Capitate glandular trichomes in *Aldama discolor* (Heliantheae – Asteraceae): morphology, metabolite profile and sesquiterpenes biosynthesis

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Abstract

The capitate glandular trichome is the most common type described to Asteraceae species. It is known for its ability to produce various plant metabolites of ecological and economical importance, among which sesquiterpenes lactones are predominant. In this paper, we applied microscopic, phytochemical and molecular genetics techniques to characterize the capitate glandular trichome found in *Aldama discolor*, a native Brazilian species of Asteraceae family, with pharmacological potential. It was found that the formation of the trichomes on leaf primordia of germinating seeds starts between 24h and 48h after radicle growth indicated germination. The beginning of the metabolic activity of the trichomes was indicated by the separation of the cuticle from the cell wall of the secretory cells at the trichome tip after 72h. This coincided with the accumulation of Budlein A, the major sesquiterpene lactone of *A. discolor* capitate glandular trichomes, in extracts of leaf primordia after 96h. In the same time frame of 72-96h post germination, gene expression studies showed an upregulation of the putative germacrene A synthase (pGAS2) and putative germacrene A oxidase (pGAO) of *A. discolor* in the transcriptome of these samples, thus indicating the beginning of sesquiterpene lactone biosynthesis. Sequencencing of the two genes revealed high similarity with HaGAS and HaGAO from sunflower, which shows that key steps of this pathway are highly conserved. The processes of trichome differentiation, metabolic activity and genetic regulation in *A. discolor* and in sunflower appears to be typical for other species of the subtribe Helianthinae.

Key-words: gene expression, leaf primordia, metabolite profile, microscopy, sesquiterpene lactones, trichome differentiation.

Introduction

Trichomes are widespread epidermal structures on vegetative and reproductive organs with great diversity in shape, cytology and function (Johnson 1975; Werker & Fahn 1981; Spring *et al.* 2000; Werker 2000). These structures provide a multitude of functions for ecological interactions and protection against biological or physical stresses (Wagner 1991; Spring *et al.* 2000). There are more than 300 types of plant trichomes described (Wagner 1991) and the Asteraceae family, with its approximately 25,000 species (Panero & Funk 2008), displays a great diversity of trichome types (Ramayya 1962; Werker & Fahn 1981; Castro *et al.* 1997; Monteiro *et al.* 2001; Mayekiso *et al.* 2008; Amrehn *et al*. 2013).

Within Asteraceae, the most reported trichome type is the capitate glandular trichome (CGT), which is common to many tribes of the family and is known to produce terpenoids, flavonoids and other resinous plant metabolites (Kelsey *et al.* 1984; Duke *et al.* 2000; Göpfert *et al.* 2009). Among the terpenoids, sesquiterpene lactones (STL) are typical secondary metabolites generally associated with the CGT of this plant family (Spring *et al.* 1987; Spring 1991; Spring 2001; Da Costa *et al*. 2005; Appezzato-da-Glória *et al.*2012), and because these compounds are mostly phytotoxic, they are usually synthesized and secreted into the subcuticular space, in the apical portion of the CGTs (Appezzato-da-Glória *et al.* 2012).

Aldama La Llave, a South American genus of Asteraceae, has 35 Brazilian species, of which 17 species are endemic and occur mainly in savanna areas (Cerrado) (Magenta *et al.* 2010). For Brazilian *Aldama* species, two types of glandular trichomes are reported: the capitate glandular trichome (CGT) and the linear glandular trichome (LGT) (Bombo *et al.* 2012; Oliveira *et al.* 2013; Silva *et al*. 2014). The predominant kind of secondary metabolites found in CGT of *Aldama* (and other taxa of the tribe Heliantheae) were STLs (Spring *et al.* 2001; Spring *et al*. 2003; Stefani *et al*. 2003; Göpfert *et al.* 2005; Appezzato-da-Glória *et al*. 2012). Some STLs were isolated and characterizes from Brazilian *Aldama* species, like guaianolides, furanoheliangolides, germacrolides, heliangolides, eudesmanolide, and also diterpenes as kaurane-type and pimarane-type can be present (Ambrósio *et al.* 2004; Schorr *et al.* 2002; Arakawa *et al.* 2008).

Although the morphology and chemistry are well known from CGT, genes involved in the trichome-based biosynthesis of STL have been identified and characterized only in *Helianthus annuus* (Göpfert 2008; Göpfert *et al.* 2009; Nguyen *et al.* 2010; Ikezawa *et al.* 2011). For native Brazilian plants, these aspects have never been approached and nothing is known about genes related to the STL production of these species, despite a survey on the secondary metabolite chemistry of the genus (Da Costa *et al.* 2001; Schorr *et al.* 2002; Ambrósio *et al.* 2004). Furthermore, for *Aldama* representatives, details of the morphology and ontogenesis of CGT have yet to be described. All these aspects could be of particular interest, because Brazilian *Aldama*species have biological and pharmacological potential, as

antimicrobial activity reported to *A. arenaria* (= *Viguiera arenaria*) (Carvalho et al. 2011), or the therapeutic potential in the control of the inflammatory process by the STL Budlein A (Arakawa *et al.* 2008; Nicolete *et al.* 2009).

This study, therefore, aimed to characterize CGT found in *Aldama discolor*, which was selected as a model for trichome development, morphology and metabolic activity in other *Aldama* species. *A. discolor* was chosen because it is an aromatic and potentially resiniferous species and represents the typical distribution of *Aldama* species in Brazil, which occur mostly in Cerrado areas (Magenta et al. 2010, 2014). Similar as recently reported for *Helianthus annuus* (Aschenbrenner *et al.* 2015), we used light, UV and scanning electron microscopic techniques to record cell differentiation, high performance liquid chromatography (HPLC) to characterize the metabolite profile, and gene expression studies to trace the activity of key enzymes of secondary metabolite pathways located in trichomes.

Materials and methods

Plant material

Seeds and axillary buds from *Aldama discolor* were collected from a field area in Minas Gerais state (geographic coordinates 19 47 01.0 S, 047 14 33.6 W), Brazil, in October, 2013. Samples from the plants that were obtained from the seed germination tests were registered and incorporated into the collection of the Herbarium HOH, Universität Hohenheim, Stuttgart, Germany (HOH 014250).

Germination tests and trichome differentiation on the leaf primordia

Differentiation and development of the trichomes was examined in leaf primordia of the *Aldamadiscolor* plants. These plants were germinated from untreated seeds following the protocol of Aschenbrenner *et al.* (2015). The samples for analyses were harvested at nine different time points representing different developmental stages from 0h to 192h after seed coating removal. Some seedlings were grown until two months old and then were harvested for HPLC analyses.

Light and fluorescence microscopy

For trichome investigations, besides the leaf primordia from the germination tests, shoot buds were collected previously from natural populations of *Aldamadiscolor*. These buds were fixed in FAA 50 (formaldehyde, acetic acid, and 50% ethanol in a 1:1:18 ratio) (Johansen 1940) and usual histological techniques were applied to the glass slides preparation. The images were digitally captured with a Leica DMLB microscope (Leica, Wetzlar, Germany) containing a video camera connected to a computer. IM50 software (Leica) was utilized for image analysis.

For trichome ontogenesis studies, serial sections (5–7 mm thick) were cut on a rotary microtome, stained using 0.05% toluidine blue in a pH 4.5 phosphate and citric acid buffer (Sakai 1973), and mounted in Entellan® synthetic resin (Merck®, Darmstadt, Germany) on glass slides.

For leaf primordia development analysis, fresh leaf primordia were harvested and the slides were prepared and analysed under light and fluorescence microscope. The images were digitally captured with a digital camera (Canon Powershot A640) connected on an Axioplan microscope (Zeiss, Oberkochen). The following fluorescence filter (Zeiss) was used: filter I: 05/395-440; excitation: 395-440 nm, beam splitter 460 nm, emission: 470 nm. IM50 software (Leica) was utilized for image analysis.

Scanning Electron Microscopy (SEM)

For SEM analyses, samples from 0h to 144h (0 to 6 days, because after this stage the leaves were totally expanded in the seedlings) were fixed in Karnovsky solution (Karnovsky 1965), dehydrated in an acetone series up to absolute acetone, dried using the critical point method with CO_2 (Horridge & Tamm 1969), mounted on aluminum stubs, and coated with a layer of 30–40 nm of gold using a Balzers SCD 050 sputter-coater. The observations and photomicrographs were obtained using a LEO 435 VP SEM (Zeiss, Oberkochen, Germany), which was operated at 20 kV, and scale bars were directly printed on the electron micrographs that were generated.

Metabolite profile

To determine metabolite profiles of leaf primordia, the same developmental stages mentioned before (0-192h) were considered. For each stage, ten leaf primordia pairs were excised from seedlings and pooled in one sample. For comparison, mature trichomes of leaves from two months old plants were also harvested. The samples were dried in environmental temperature for two days, extracted with 25 µL of acetonitrile and five minutes in an ultrasonic bath. The plant tissue was removed by centrifugation (2 min, 15,000 rpm) and the supernatants were adjusted to 25% acetonitrile by adding ddH_2O. Free-hand collection of CGT from dried leaves was also performed. The collected trichomes were transferred directly into acetonitrile, sonicated (5 min ultrasonic bath), centrifuged (2 min, 15,000 rpm) and the supernatant was directly used for chemical analyses.

Compound identification

Compound identification was done by comparison with previous studies and by fractionation of the extracts after separation by high performance liquid chromatography (HPLC). Six main fractions were dried in a vacuum centrifuge, dissolved with deuterated chloroform ($CDCl_3$), and analyzed by ^1H nuclear magnetic resonance (NMR) for structural

elucidation, on a Unity Inova spectrometer (Varian GmbH, Darmstadt) with 500 MHz in a PFGID sample head.

High Performance liquid chromatography

The high performance liquid chromatography was carried out on a P580 liquid chromatographic system (Dionex P580 A HPG series; Dionex Softron GmbH; column: ODS Z2 Kromasil 120 ST, 3 µm; 150 x 4.6 mm). As an eluent, a gradient of acetonitrile-water was chosen (25-45% in 30 min, 45-100% to 45 min; flow rate: 1 mL/min and 25-45% in 30 min, 45-100% to 31 min; flow rate: 0.8 mL/min). Detection was performed with a UV/VIS detector (Dionex UVD 340S; Dionex Softron GmbH) controlled by the software Chromeleon (Chromeleon Peak Net Version 6.01; Dionex Corporation).

Total RNA extraction and cDNA synthesis

For RNA extraction, three different developmental stages, corresponding to 24h, 72h and 144h were chosen. For each stage, 15 pairs of primary leaf primordia were harvested from fresh plants and pooled for RNA extraction as described previously (Aschenbrenner *et al.* 2015).

The harvested samples were transferred to RNAse-free tubes, immediately frozen in liquid nitrogen and maintained at -70 °C until further use. For total RNA extraction, the EURx GeneMATRIX Universal RNA Purification Kit (Roboklon GmbH, Berlin, Germany) was applied. To eliminate residual genomic DNA from total RNA, PerfeCta® Dnase I (Rnase-free) kit (Quanta Biosciencies) was used, both according to the manufacturer's instructions. The quantity and integrity of the isolated RNA was verified with the Bioanalyzer 2100 using a RNA 6000 Pico Chip (Agilent, Böblingen, Germany). For PCR reactions, cDNA synthesis was performed using the ReveredAid cDNA synthesis kit (Fermentas, St.Leon-Roth, Germany). All steps were carried out as described in the manual.

Semi-quantitative PCRs and expression of genes of the secondary metabolism

To investigate the association among trichome differentiation, metabolite profile and the expression of genes related to secondary metabolism in leaf primordia, the differential expression of genes of the flavonoid, sesquiterpene and STL pathway was determined by, semi-quantitative PCR. Ubiquitin was used as housekeeping gene (reference gene), and the amplification intensity obtained for each gene of interest was compared relative to the amplification of the reference gene.

The investigated genes from the secondary metabolism were chosen according to Aschenbrenner *et al.* (2015). The specific oligonucleotide primers and annealing temperatures for PCR are given in Table 1. PCR reactions were performed in a peqSTAR Thermocycler (Peqlab, Erlangen, Germany). For the amplification reactions, 0.2 mL tubes (Sarstedt AG, Nümbrecht) and DNase and RNase-free water were used. All PCR experiments were carried out

with RedTaq MasterMix (Genaxxon Bioscience, Ulm, Germany). The PCR conditions were 4 min initial denaturation followed by 36 cycles of 20 s denaturation (95 ºC), 20 s annealing (X ºC; see Table 1), 1 min/kb elongation (72 ºC) and final elongation for 4 min (72 ºC).

For the evaluation of the semi-quantitative PCRs, capillary electrophoresis was performed in a microchip electrophoresis MCE220 (Shimadzu Corporation, Kyoto, Japan) coupled to the MultiNA Control and MultiNA Viewer softwares, and different buffers and ladder systems were used depending on the product size (Shimadzu DNA 500 kit, 1000 kit, and 2500 kit, Shimadzu Corporation, Kyoto). Two biological repetitions with each three technical repetitions were carried out for each detected gene.

For statistical analysis, means of each experiment were used for ANOVA with $p > 0.05\%$ as significance level.

Fragments sequencing and analyzes of the results

All the PCR fragments were sequenced by Macrogen (Amsterdam, Netherlands). For sequence comparison, the results were analysed using BLAST (Basic Local Alignment Search Tool), available in http://www.ncbi.nlm.nih.gov/BLAST. The software Bioedit Sequence Alignment Editor (Hall, 1999) was used in order to processing the sequences.

Results

Capitate glandular trichome ontogenesis

Aldama discolor capitate glandular trichomes were observed on leaves and stems of seedlings and adult plants. The development of these trichomes occurred already on the leaf primordia and it was not synchronized, i.e., there were trichomes in different differentiation stages located side by side on the primordial leaves (Figure 1A). However, when the development of the leaf was completed, all trichomes were almost in the same developmental stage.

The CGT develops from a single protodermic cell (Figure 1B) that divides anticlinally, giving rise to a 2-cells stage. Afterwards, both cells divide periclinally to form the four-cell stage (Figure 1C). Subsequently, a number of periclinal divisions of the cells follow until reaching the final stage, which contains 14 cells (Figures 1D-H). The fully developed trichome is constituted by seven pairs of cells: one pair of basal cells, four cell pairs that constitute the stalk, and the upper two pairs that constitute the secretory head cells (Figure 1H). A lipophilic secretion accumulates in the subcuticular space (Figure 1H). During the secretion process, the cuticle extends until it may break open to release the exudate. The cells of mature trichomes collapse once the secretion has been finished (Figure 1I).

CGT differentiation on the leaf primordia

The development of the capitate glandular trichomes was monitored with light (LM), fluorescence (FM) and scanning electron microscopy (SEM) at different leaf primordia stages. In the first two stages (0h, 24h), no CGT were found; they first became visible after 48h, when the stalk cells had developed (Figures 2A,E).

In the 72h (Figures 2B,F,I) and 96h (Figures 2C,G,J) stages, CGT increased in cell number and started to expand the cuticular globe on top of the apical cells, respectively. There was a differentiation among the cell layers: two to four of the apical cells pairs were transparent in LM investigations and bluish under UV light (Figure 2F,G), while the basal cells pair exhibit green color in LM and red color in UV light due to the presence of chloroplasts.

At the 120h stage (Figures 2D,H), CGT exhibited a fully expanded cuticle is visible. The disruption of the cuticle occurred along a predetermined line between the two cells at the top of the head of the trichome (Figure 2K) and caused the release of the exudates (Figure 2L).

Metabolite profiles from different stages of leaf primordia and compound identification

Extracts of fully developed leaves and also selectively harvested CGTs from leaves at the same development level were analyzed with HPLC to characterization of the metabolites accumulated in these trichomes (Figure 3). It was possible to identify Budlein A (main peak, retention time: 11.5 min; UV maximum absorption (nm) 217.26), which was confirmed by comparison with an authentic reference sample as well as by ^{1}H NMR analysis of a purified sample from this peak, which showed spectral identity with literature data. No structures were assigned to the compounds of the others peaks.

In the same way, extracts of leaf primordia from different stages were examined in order to monitor the accumulation of metabolites by the CGT along their development (Fig. 4). No specific compound of the CGT appeared in extracts of the primordia up to 72h. Budlein A was found in extracts of primordia at stage 96h and onwards. At this time the cuticular globe starts to expand. The amount of budlein A gradually continued to be accumulated until 168h stage.

Expression of genes of the secondary metabolism

In order to detect gene expression related to trichome-based STL and flavonoid biosynthesis, a semi-quantitative analysis was made based on transcribed genes in three different stages of the leaf primordia and normalized to ubiquitin, which was used as housekeeping gene. The investigated genes were related to the isoprenoid biosynthesis (FPPS), flavonoid pathway (CHS), and the genes related to the sesquiterpenes lactones biosynthesis, germacrene A synthase (GAS2) and germacrene A acid oxidase GAO) (Figure 5).

All primers used for the gene expression studies, except for the Ubiquitin gene, were originally designed for *Helianthus annuus* genes. Despite of this, the amplification was achieved and sequencing proved high homology of the sequences from *Aldama discolor* thus confirming the close phylogenetic relationship between the two genera andindicating the same function in *Aldama* as in *Helianthus*. All the PCR products were sequenced and their identities were confirmed by Blast searches (*www.ncbi.nlm.nih.gov/genbank)*. The sequence identities with *Helianthus annuus* were: 98% for FPPS, GAS2 and GAAO, and 99% for GAO and 96% for CHS (see supplementary data).

For the putative chalcone synthase (pCHS) of *Aldama*, a similar expression rate was observed in all three stages and no increase was observed. Similarly, farnesylpyrophosphate synthase (pFPPS) showed an equal and relatively low expression (when compared to the other genes) in all three stages (Figure 5). In contrast, the expression of a putative germacrene A synthase 2 (pGAS2) was not detected in samples of the 24h stage, but was found at the 72h stage, and more evidently after 144h. This upregulation was even more evident with the putative germacrene A oxidase (pGAO), the second enzyme involved in the synthesis of STL from FPP (Figure 5). The primers from *H. annuus* specific for germacrene A acid oxidase (GAAO) were also tested, but gave no amplification product with cDNA of *A. discolor* (data not shown)

Discussion

The capitate glandular trichome described here for *A. discolor* presented the same anatomical features as already reported for other *Aldama* species (Bombo *et al.* 2012; Oliveira *et al.* 2013; Silva *et al.* 2014). In addition, the investigations of CGT in *A. discolor* have shown many typical characteristics of biseriate glandular trichomes found in other Asteraceae genera (Monteiro *et al.* 2001; Appezzato-da-Glória *et al.* 2012; Amrehn *et al.* 2013). Alike shown for some Asteraceae species from other tribes (Monteiro *et al.* 2001), the development of CGT is not synchronized on the leaf primordia in *A. discolor*, and there are trichomes in distinct differentiation stages located side by side. This is different from the situation in *Helianthus annuus*, a close relative to *Aldama*, where mature and immature trichomes were simultaneously observed on the same floret or leaf (Amrehn *et al.* 2013). However, in later stages of leaf development, all CGT in *A. discolor* were fully developed thus indicating that, like in sunflower (Aschenbrenner *et al.* 2015), their formation is restricted to a very early stage of the organogenesis.

The trichomes were observed under UV light, in order to characterize the autofluorescence in their cells. Autofluorescence could be diagnostic to flavonoids, and the color yellow, green or blue under UV-365 nm light, depends on the structural type (Wagner &

Bladt 1996). Göpfert *et al.* (2009), by using a 440 nm filter, found a blue fluorescence in the CGT of flowering *H. annuus* exposed to sunlight conditions, caused by the presence of the flavonoid 5-dehydro-nevadensin. For *A. discolor*, there was no clear indication for the presence of flavonoids in the cuticular globe of the plants tested, whereas in mature trichomes from older plants, strong bluish fluorescence was found (data not shown). This may be explained by the cultivation conditions in the climate chamber, where low light intensity was not inductive to the production of flavonoids (Aschenbrenner *et al*. 2015), which usually functions as protection against UV irradiation (Spring *et al.* 1989; Lusa *et al.* 2015).

Concerning the metabolite profile from the leaf primordia in different developmental stages, the two main peaks observed on the chromatograms were associated to the CGT, as was indicated by similar peak retention times and identical UV spectra with chromatograms from manually isolated mature CGT. After 96h of plant development the increase of metabolite peaks showed the transition of CGT from the presecretory to the secretory phase, thus coinciding with the beginning of the cuticular globe expansion. The STL budlein A, a compound that has already been isolated from different *Aldama* (*Viguiera*) species (Vivar *et al*. 1976; Valerio *et al*. 2007; Arakawa *et al.* 2008; Nicolete *et al*. 2009) and have a therapeutic potential in the control of the inflammatory process (Nicolete *et al.* 2009), was identified as the major peak in both CGT and leaf primordia extracts.

Regarding the gene expression analyses, specific key enzymes related to the flavonoid and sesquiterpene metabolism were investigated. CHS is a key enzyme of the flavonoid biosynthesis and can be influenced by stress and environmental factors, such as UV light incidence, wounding or pathogen attack (Dao *et al.* 2011). A low relative expression of this enzyme was detected in all three stages and no differences were observed among them, as reported to *Helianthus annuus* (Aschenbrenner *et al*. 2015). This low expression seems to be a consequence of the experimental conditions, because the plants were cultivated under low intensity light. The results were in agreement with the UV analyses where there was no clear indication for flavonoids presence accumulation into the CGTs.

Farnesylpyrophosphate synthase (FPPS) showed a relatively low expression in all three stages. This enzyme is a key to a wide range of terpenoids in the plant metabolism (Zulak & Bohlmann 2010; Chen *et al.* 2011), including compounds that are required for growth and development, such as phytosterols (membrane structure and function), dolichols (glycoprotein synthesis), ubiquinones and on the cell proliferation (Cunillera *et al.* 1996; Chen *et al.* 2011; Aschenbrenner 2014). Hence, a fairly constant level of expression was expected.

Concerning the genes exclusively associated with the STL biosynthesis and therefore expected to be correlated with CGT development, we saw an upregulation of the pGAS2 gene, and particularly of the pGAO gene in the trichome stage after 72h. Germacrene A synthase catalyzes the first step of STL biosynthesis by folding FPP into germacrene A (Göpfert

et al. 2009), whereas germacrene A oxidase is involved in the formation of germacrene A acid (Nguyen *et al.* 2010) which is then hydroxylated at C6 position to give costunolide as a first basic STL (Ikezawa *et al.* 2011). Thus we found, that the trichomes of *A. discolor* start their STL biosynthesis shortly after cellular differentiation has been accomplished and that this metabolic activity lasts over several days. This coincided with the expansion of the cuticular globe, in which the produced STL are stored, and with the appearance of budlein A in samples of the 96h stage. The results showed the same tendency of expression as in sunflower trichomes of *H. annuus* (Aschenbrenner *et al.* 2015), except for germacrene A acid oxigenase (GAAO), for which no relative expression was detected due to the lack of suitable primers.

The high sequence identities between *A. discolor* and *H. annuus* shown in the blast experiments and the similarity on relative gene expression between these two species corroborate the results of Nguyen *et al.* (2010), who demonstrated that GAOs and their corresponding enzymatic activity are highly conserved in Asteraceae representatives from different clades. In its turn, any relative expression was detected for the putative gene germacrene A acid oxidase (data not shown), which indicates that this gene, or the specific region used here, could be more altered in *A. discolor*, when compared to *H. annuus* although the C-8 hydroxylation, catalyzed by GAAO (Ikezawa *et al.* 2011), also occurs in *Aldama*, demonstrated here with the presence of the Budlein A.

In this study, we successfully employed a model system from sunflower (Aschenbrenner *et al.* 2015) for the investigation of trichome development in *Aldama* species. The results showed, that the processes of trichome differentiation, metabolic activity and genetic regulation known from *H. annuus*, a species that has several aspects extensively investigated (Spring *et al.* 1992; Göpfert *et al.* 2005; 2009; Ikezawa *et al.* 2011; Amrehn *et al.* 2013; Aschenbrenner *et al.* 2013; 2015), also account for *A. discolor* and most likely other species of the subtribe Helianthinae. This was the first time that such an approach was applied to a native Brazilian Asteraceae species, for which no information about its genomic and molecular traits was available.

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References

Ambrósio S., Schorr K., Da Costa F. (2004) Terpenoids of *Viguiera arenaria* (Asteraceae). *Biochemical Systematics and Ecology*, **32**(2), 221-224.

Amrehn E., Heller A., Spring O. (2014) Capitate glandular trichomes of *Helianthus annuus* (Asteraceae): ultrastructure and cytological development. *Protoplasma*, **251**(1), 161-167.

Appezzato-da-Glória B., Da Costa F.B., Silva V.C.d., Gobbo-Neto L., Rehder V.L.G., Hayashi A.H. (2012) Glandular trichomes on aerial and underground organs in *Chrysolaena* species (Vernonieae – Asteraceae): Structure, ultrastructure and chemical composition. *Flora*, **207**, 878-887.

Arakawa N., Schorr K., Ambrósio S., Merfort I., Da Costa F. (2008) Further sesquiterpene lactones from *Viguiera robusta* and the potential anti-inflammatory activity of a heliangolide: Inhibition of human neutrophil elastase release. *Zeitschrift Fur Naturforschung Section C - A Journal of Biosciences*, **63**(7-8), 533-538.

Aschenbrenner A.K. (2014) *Die linearen Drüsenhaare der Sonnenblume-Morphologie, Verbreitung, Metabolitprofil und Sesquiterpenbiosynthese*. PhD thesis, Institute of Botany, University of Hohenheim.

Aschenbrenner A.K., Amrehn E., Bechtel L., Spring O. (2015) Trichome differentiation on leaf primordia of *Helianthus annuus* (Asteraceae): morphology, gene expression and metabolite profile. *Planta*, **241**(4), 837-846.

Aschenbrenner A.K., Horakh S., Spring O. (2013) Linear glandular trichomes of *Helianthus* (Asteraceae): morphology, localization, metabolite activity and occurrence. *AoB Plants*, **5**, plt028.

Bennett M.H., Mansfield J.W., Lewis M.J., Beale M.H. (2002) Cloning and expression of sesquiterpene synthase genes from lettuce (*Lactuca sativa* L.). *Phytochemistry*, **60**(3), 255-261.

Bombo A., Oliveira T., Oliveira A., Rehder V., Magenta M., Appezzato-da-Gloria B. (2012) Anatomy and essential oils from aerial organs in three species of *Aldama* (Asteraceae-Heliantheae) that have a difficult delimitation. *Australian Journal of Botany*, **60**(7), 632-642.

Bouwmeester H.J., Kodde J., Verstappen F.W., Altug I.G., Kraker J.W., Wallaart T.E. (2002) Isolation and characterization of two germacrene A synthase cDNA clones from chicory. *Plant Physiology*, **129**(1), 134-144.

Carvalho T., Simao M., Ambrósio S., Furtado N., Veneziani R., Heleno V., Da Costa F., Gomes B., Souza M., Reis E., Martins C. (2011) Antimicrobial Activity of Diterpenes from *Viguiera arenaria* against Endodontic Bacteria. *Molecules*, **16**(1), 543-551.

Castro M.D.M., Leitão-Filho H.D.F., Monteiro W.R. (1997) Utilização de estruturas secretoras na identificação dos gêneros de Asteraceae de uma vegetação de cerrado. *Revista Brasileira de Botânica*, **20**(2), 163-174.

Chen F., Tholl D., Bohlmann J., Pichersky E. (2011) The family of terpene synthases in plants: a mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. *The Plant Journal*, **66**(1), 212-229.

Cunillera N., Arró M., Delourme D., Karst F., Boronat A., Ferrer A. (1996) *Arabidopsis thaliana* contains two differentially expressed farnesyl-diphosphate synthase genes. *The Journal of Biological Chemistry*, **271**(13), 7774-7780.

Da Costa F.B.; Vichnewski W.; Herz W. (1996) Constituents of *Viguiera aspilioides* and *V. robusta*. *Biochemical Systematics and Ecology*, **24**(6), 585-587.

Da Costa F.B., Terfloth L., Gasteiger J. (2005) Sesquiterpene lactone-based classification of three Asteraceae tribes: a study based on self-organizing neural networks applied to chemosystematics. *Phytochemistry*, **66**(3), 345-353.

Dao T.T., Linthorst H.J., Verpoorte R. (2011) Chalcone synthase and its functions in plant resistance. *Phytochemistry Reviews*, **10**(3), 397-412.

Duke S.O., Canel C., Rimando A.M., Tellez M.R., Duke M.V., Paul R.N. Current Potential (2000) Exploitation of Plant Glandular Trichome Productivity. In: Hallahan D.L., Gray J.C. (Eds), *Plant Trichomes*, Academic Press, San Diego, USA: 121-152.

Göpfert J., Heil N., Conrad J., Spring O. (2005) Cytological development and sesquiterpene lactone secretion in capitate glandular trichomes of sunflower. *Plant Biology*, **7**(2), 148-155.

Göpfert J.C. (2008) *Molekulare und entwicklungsbiologische Charakterisierung von Schlüsselenzymen der Naturstoffbiosynthese in Drüsenhaaren der Sonnenblume*. PhD thesis, Institute of Botany, University of Hohenheim, Stuttgart.

Göpfert J.C., Macnevin G., Ro D.K., Spring O. (2009) Identification, functional characterization and developmental regulation of sesquiterpene synthases from sunflower capitate glandular trichomes. *BMC Plant Biology*, **9**, 86.

Hall T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95-98.

Horridge G., Tamm, S. (1969) Critical point drying for scanning electron microscopic study of ciliary motion. *Science*, 817-818.

Ikezawa N., Göpfert J.C., Nguyen D.T., Kim S.U., O'Maille P.E., Spring O., Ro D.K. (2011) Lettuce costunolide synthase (CYP71BL2) and its homolog (CYP71BL1) from sunflower catalyze distinct regio- and stereoselective hydroxylations in sesquiterpene lactone metabolism. *The Journal of Biological Chemistry*, **286**(24), 21601-21611.

Johansen D. (1940) *Plant microtechnique*. McGraw-Hill Book Co. Inc., New York, USA: 523 pp.

Johnson H. (1975) Plant Pubescence - Ecological Perspective. *Botanical Review*, **41**(3), 233-258.

Karnovsky M.J. (1965) A Formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *The Journal of cell Biology*, **27**, 137-138A.

Kelsey R. (1984) Glandular Trichomes - a helpful taxonomic character for *Artemisia nova* (Black Sagebrush). *Journal of Range Management*, **37**(4), 370-372.

Kim M.Y., Chang Y.J., Bang M.H., Baek N.I., Jin J., Lee C.H., Kim S.U. (2005) cDNA isolation and characterization of (+)-germacrena A synthase from *Ixeris dentata* form. *albiflora* Hara. *Journal of Plant Biology*, **48**(2), 178-186.

Lusa M.G. (2014) *Morfoanatomia e fitoquímica de espécies da subtribo Lychnophorinae (Asteraceae: Vernoniae) como subsídios para as análises filogenéticas do grupo*. PhD thesis, University of Campinas, Campinas.

Magenta M.A.G., Pirani J.R. (2014) Novidades taxonômicas em *Aldama* (Asteraceae-Heliantheae). *Rodriguésia***, 65**(1), 175-192.

Magenta M.A.G., Pirani J.R., Mondin C.A. (2010) Novos táxons e combinações em *Viguiera* (Asteraceae - Heliantheae). *Rodriguésia*, **61**(1), 1-11.

Marquina S., Maldonado N., Garduno-Ramirez M., Aranda E., Villarreal M., Navarro V., Bye R., Delgado G., Alvarez L. (2001) Bioactive oleanolic acid saponins and other constituents from the roots of *Viguiera decurrens*. *Phytochemistry*, **56**(1), 93-97.

Mayekiso B., Magwa M.L., Coopoosamy R. (2008) The morphology and ultrastructure of glandular and non-glandular trichomes of *Pteronia incana* (Asteraceae). *African Journal of Plant Science*, **2**(7), 52-60.

Monteiro W.R., Castro M.M., Mazzoni-Viveiros S.C., Mahlberg P.G. (2001) Development and some histochemical aspects of foliar glandular trichomes of *Stevia rebaudiana* (Bert.) Bert. – Asteraceae. *Revista Brasileira de Botânica*, **24**(3), 349-357.

Nguyen D.T., Göpfert J.C., Ikezawa N., Macnevin G., Kathiresan M., Conrad J., Spring O., Ro D.K. (2010) Biochemical conservation and evolution of germacrene A oxidase in Asteraceae. *The Journal of Biological Chemistry*, **285**(22), 16588-16598.

Nicolete R., Arakawa N.S., Rius C., Nomizo A., Jose P.J., Da Costa F.B., Sanz M.-J., Faccioli L.H. (2009) Budlein A from *Viguiera robusta* inhibits leukocyte-endothelial cell interactions, adhesion molecule expression and inflammatory mediators release. *Phytomedicine*, **16**(10), 904-915.

Oliveira T.S.d., Bombo A.B., Appezzato-da-Glória B. (2013) Anatomy of vegetative organs with an emphasis on the secretory structures of two species of *Aldama* (Asteraceae – Heliantheae). *Botany*, **91**, 335-342.

Panero J., Funk V. (2008) The value of sampling anomalous taxa in phylogenetic studies: Major clades of the Asteraceae revealed. *Molecular Phylogenetics and Evolution*, **47**(2), 757-782.

Ramayya N. (1962) Studies on the trichomes of some Compositae II. Phylogeny and Classification. *Bulletin of the Botanical Survey of India*, **4**, 177-188.

Sakai w. (1973) simple method for differential staining of paraffin embedded plant material using Toluidine blue O. *Stain Technology*, 247-249.

Schorr K., Garcia-Piñeres A.J., Siedle B., Merfort I., Da Costa F.B. (2002), Guaianolides from *Viguiera gardneri* inhibit the transcription factor NF-*κ*B. *Phytochemistry*,**60**, 733-740.

Silva E.M.S., Hayashi A.H., Appezzato-da-Glória B. (2014) Anatomy of vegetative organs in *Aldama tenuifolia* and *A. kunthiana* (Asteraceae: Heliantheae). *Brazilian Journal of Botany*, **37**(4), 505-517.

Spring O.; Bienert U.; Klemt V. (1987) Sesquiterpene lactones in glandular trichomes of sunflower leaves. *Journal of Plant Physiology*, **130**, 433-439.

Spring O.; Benz T.; Ilg M. (1989) Sesquiterpene lactones of the capitate glandular trichomes of *Helianthus annuus*. *Phytochemistry*, **28**, 745-749.

Spring O. (1991) Trichome microsampling of sesquiterpene lactones for the use of systematic studies. *Recent Advances in Phytochemistry*, **25**, 319-345.

Spring O., Rodon U., Macias F. (1992) Sesquiterpenes from noncapitate glandular trichomes of *Helianthus annuus*. *Phytochemistry*, **31**(5), 1541-1544.

Spring O., Zipper R., Klaiber I., Reeb S., Vogler B. (2000) Sesquiterpene lactones in *Viguiera eriophora* and *Viguiera peruana* (Heliantheae; Asteraceae). *Phytochemistry*, **55**(3), 255-261.

Spring O., Zipper R., Reeb S., Vogler B., Da Costa F. (2001) Sesquiterpene lactones and a myoinositol from glandular trichomes of *Viguiera quinqueremis* (Heliantheae; Asteraceae). *Phytochemistry*, **57**(2), 267-272.

Spring O., Zipper R., Conrad J., Vogler B., Klaiber I., Da Costa F. (2003) Sesquiterpene lactones from glandular trichomes of *Viguiera radula* (Heliantheae; Asteraceae). *Phytochemistry*, **62**(8), 1185-1189.

Stefani R., Eberlin M.N., Tomazela D.M., Da Costa F.B. (2003) Eudesmanolides from *Dimerostemma vestitum*. *Journal of Natural Products*, **66**(3), 401-403.

Valerio D., Cunha T., Arakawa N., Lemos H., Da Costa F., Parada C., Ferreira S., Cunha F., Verri W. (2007) Anti-inflammatory and analgesic effects of the sesquiterpene lactone budlein A in mice: Inhibition of cytokine production-dependent mechanism. *European Journal of Pharmacology*, **562**(1-2), 155-163.

Vivar A.R., Guerrero C., Díaz E., Brattoef E.A., Jiménez L. (1976) The germacranolides of *Viguiera buddleiaeformis* stryctures of budlein-A and -B. *Phytochemistry*, **15**(4), 505-517.

Wagner G. (1991) Secreting glandular trichomes - more than just hairs. *Plant Physiology*, **96**(3), 675-679.

Wagner H., Bladt S. (1996) *Plant drug analysis: a thin layer chromatography atlas*. Springer-Verlag, Berlin, Germany: 384 pp.

Werker E. (2000) Trichome diversity and development. In: Hallahan D.L., Gray J.C., Callow J.A. (Eds) *Advances in Botanical Research, incorporating advances in Plant Pathology*. Academic Press, San Diego, USA: **31**, 1-35.

Werker E., Fahn A. (1981) Secretory Hairs of *Inula viscosa* (L.) Ait. - Development, Ultrastructure, and Secretion. *Botanical Gazette*, **142**(4), 461-476.

Zulak K., Bohlmann J. (2010) Terpenoid Biosynthesis and Specialized Vascular Cells of Conifer Defense. *Journal of Integrative Plant Biology*, **52**(1), 86-97.

Table 1: Investigated genes, oligonucleotide primer sequences and PCR conditions - annealing temperature (A) and elongation time (E) - for amplification of ubiquitin genes and genes related to secondary metabolism and their reference.

Gene	Description	Sequence 5' – 3'	PCR conditions	NCBI acessionnumber/ Reference
Ubiq_Aldama	Ubiquitin designed to *Aldama discolor*	F-TCCCACCAGACCAGCAGCGAT	A: 63 °C	
		R-TCACGAAGACGGAGGACGAGATG	E: 15"	
HaFPPS	HaFPPS - Farnesyl diphosphate synthase gene *Helianthus annuus*	F-ACTGCTTGTACGGCTTTGCTTG	A: 60 ºC	Aschenbrenner et al. 2015
		R-TTTCTTGCATCTGCCCTTGGTTG	E: 30"	Aschenbrenner et al. 2015
HaCHS	HaCHS - Chalcone synthase gene *H. annuus*	F-GTGTGCATACAATGCCCCGTC	A: 58 °C	Göpfert 2008
		R-CATAAACCGCTTGACCGAAGACC	E: 15"	Göpfert 2008
HaGAS2	HaGAS2 - Sesquiterpene synthases gene *H. annuus*, germacrene A synthase	F-ACGAGGGGTGTCTCAAGCCA	A: 60 °C	Spring, personal comm.
		R-GGGTGAAGAACCAACAAACAGGAGGG	E: 30"	Spring, personal comm.
HaGAO	HaGAO - Sesquiterpene synthases gene *H. annuus*, Germacrene A oxidase	F-ATGGACTTAGCCCGAAAATACG	A: 54 °C	Aschenbrenner et al. 2015
		R-GAAAGATATCCGCCACATCAAAC	E: 15"	Aschenbrenner et al. 2015
HaGAAO	HaGAAO - Sesquiterpene synthases gene *H. annuus*, Germacrene A acid oxidase	F-CGAAACCTTAGAAACTTGGTC	A: 51 °C	Aschenbrenner et al. 2015
		R-AATAAACTGTCGGTCTTCGCTAAC	E: 30"	Aschenbrenner et al. 2015

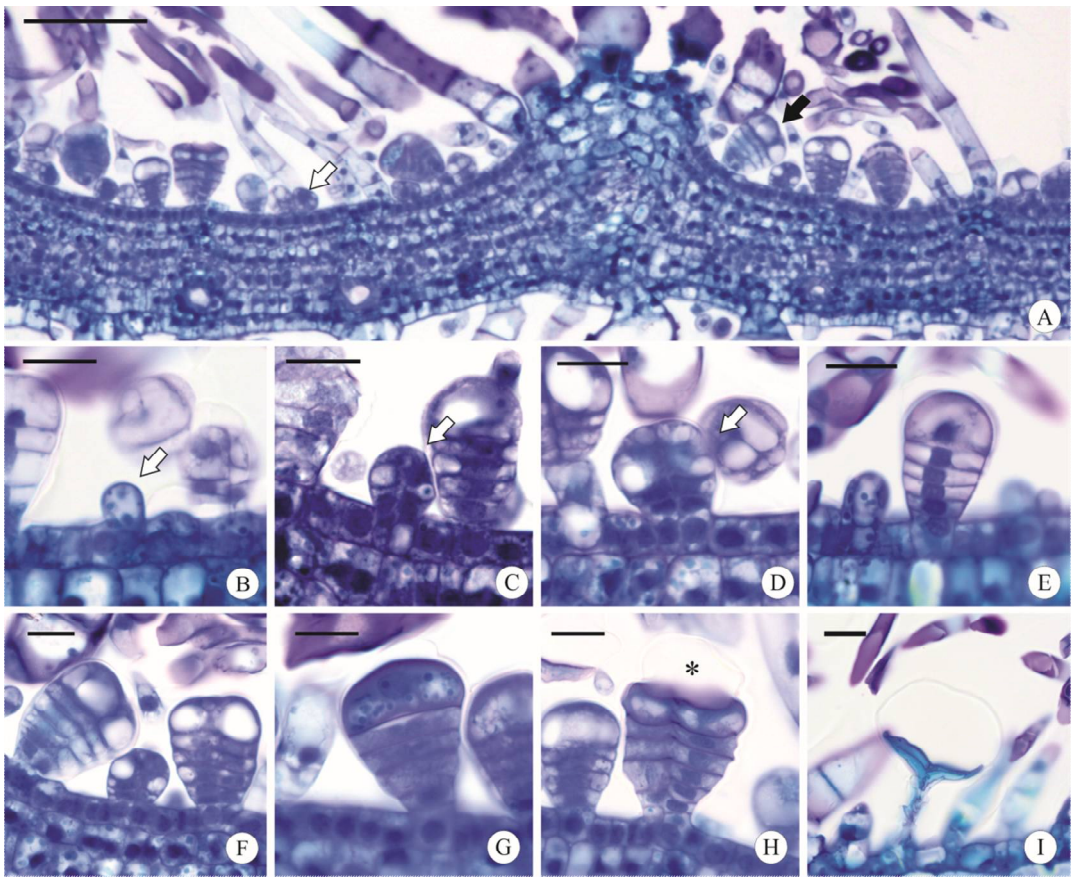

Figure 1. Longitudinal sections of the leaf primordia of *Aldamadiscolor*, measuring ca. 1.2 mm. A. General view, showing trichomes in different developmental stages. Early (white narrow) and late (black narrow) stages. B-I. Ontogenesis of the capitate glandular trichomes. (*) in H indicates the subcuticular space where the secretion is accumulated. Scale bars: A = 50 µm; B-I = 10 µm.

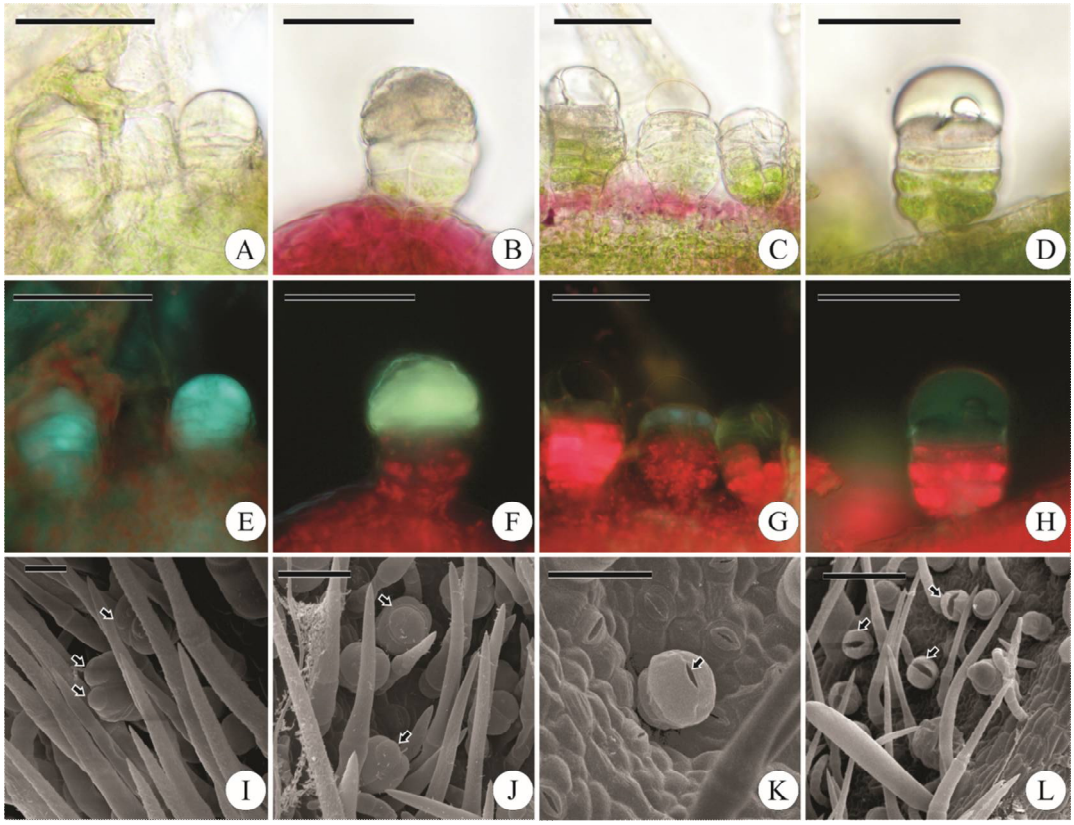

Figure 2. Capitate glandular trichome differentiation on leaf primordia of *A. discolor* under light microscopy (A-D), fluorescence microscopy with UV filter (E-H) and scanning electron microscopy (I-L). A,E. 48h stage. B,F,I,J. 72h stage. C,G. 96h stage. D,H,K. 120h stage. L. After cuticle rupture and metabolite secretion on fully expanded leaves. Expanding cuticle (arrows in I and J) and its rupture (arrows in K and L). Scale bars: A-H, J-K = 50 µm; I = 200µm; L = 100 µm.

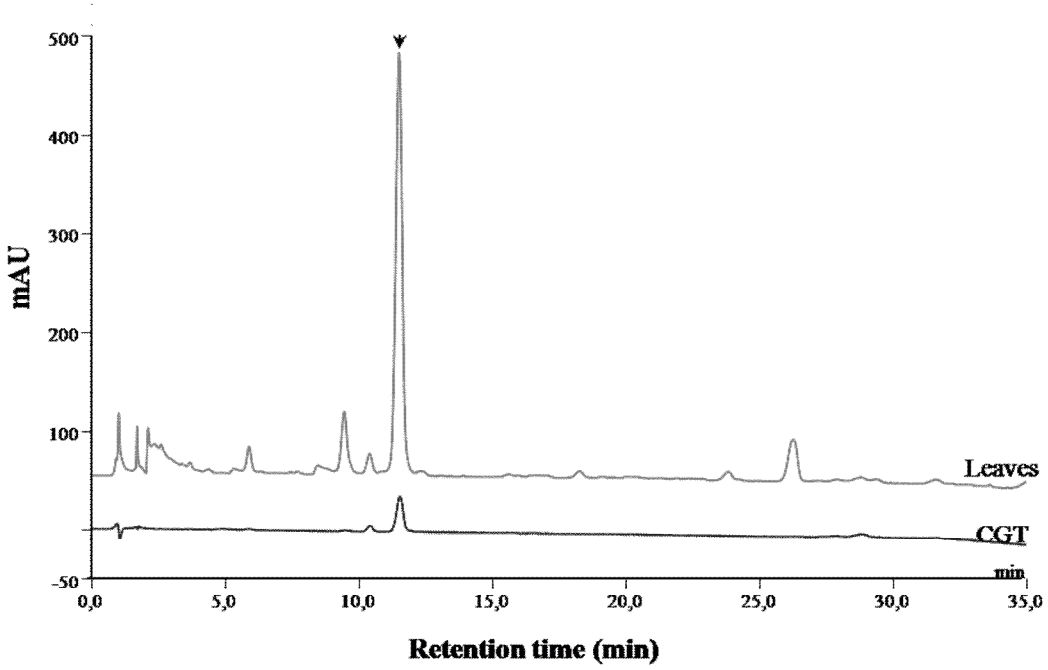

Figure 3. Metabolite profile from HPLC analyses of fully developed leaves (upper line) and manually collected CGTs from adult plants. Arrows indicate the main compound Budlein A (retention time: 11.5 min; UV maximum absorption (nm) 217; 268). WVL: 210 nm.

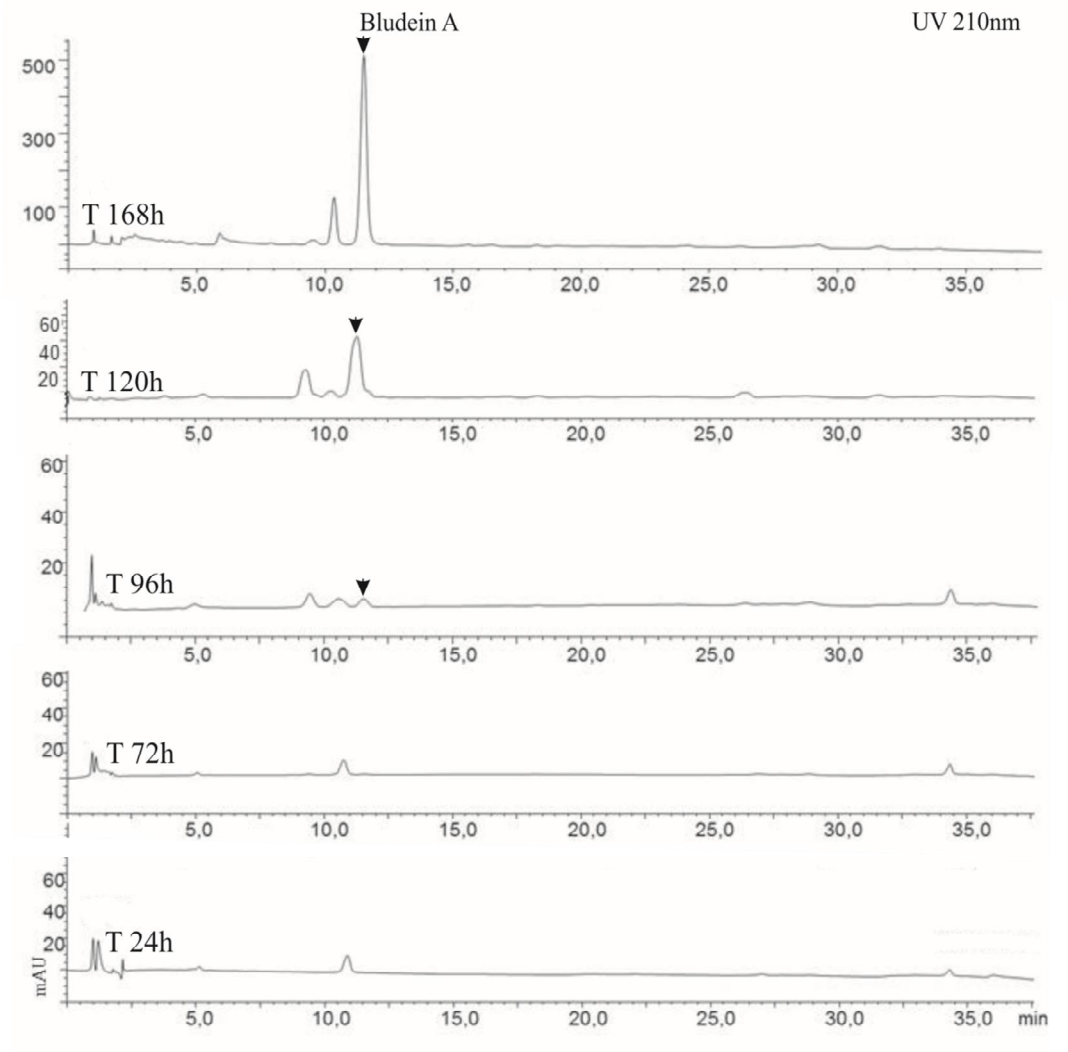

Figure 4. Time frame comparing the metabolite profile from HPLC analyses from five development stages of the leaf primordia of *Aldama discolor*. Arrows indicate the main peak, which corresponds to Budlein A (retention time: 11.5 min; UV maximum absorption (nm) 217; 268). WVL: 210 nm.

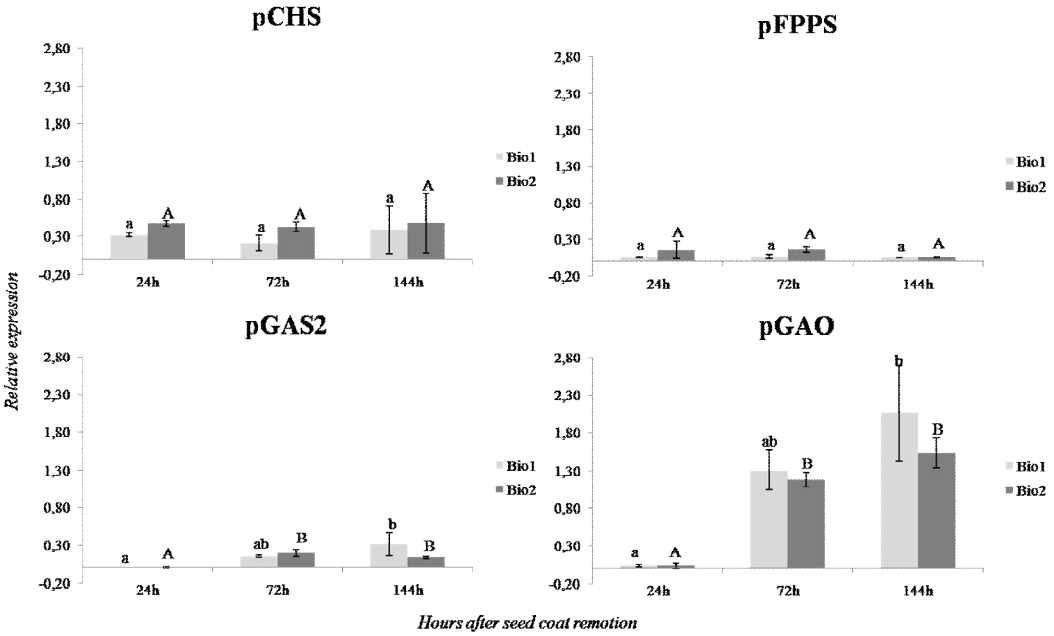

Figure 5: Means of the relative expression of the putative genes CHS, FPPS, GAS2 and GAO, in relation to the expression of the reference gene Ubiquitin, in different stages of development of the leaf primordia (24h: trichomes absent, 72h: trichomes in differentiation and 144h: trichomes completely developed, secretory phase), considering two biological repetition. Same letters into the gene graph do not differ statistically.

CONSIDERAÇÕES FINAIS DA TESE

Os estudos anatômicos têm permitido resolver diversos problemas taxonômicos em diferentes grupos de espécies e famílias, inclusive para a família Asteraceae. Dentro do grupo *Aldama*, os estudos têm auxiliado na resolução de grupos específicos de espécies, e para as quatro espécies aqui avaliadas, os resultados anatômicos trouxeram informações relevantes para a delimitação das mesmas, bem como para seu reconhecimento dentro do grupo. Algumas das características apresentadas para as espécies foram comuns à outras espécies já estudadas pelo nosso grupo de pesquisa, e mostram que há uma consistência anatômica entre as espécies brasileiras do gênero.

Também foram apresentados aspectos relacionados à produção de óleos essenciais, que têm se mostrado importantes entre alguns representantes brasileiros, devido ao potencial biológico associado a esses óleos. Alguns testes realizados pelo grupo de pesquisa têm evidenciado que os óleos de algumas espécies podem ter atividade antiproliferativa *in vitro*, ou seja, são potencialmente ativos contra células cancerígenas. Dessa forma, a identificação dos órgãos e espécies que apresentam maiores rendimentos de óleos, bem como da sua composição química, são de extrema importância, pois podem orientar estudos futuros com espécies potenciais. Entre as espécies desse estudo, duas tiveram rendimento de óleo significativo quando comparadas às demais: *Aldama grandiflora*, e *Aldama squalida*. Além do enfoque químico, também foram identificadas as estruturas secretoras responsáveis pela produção do óleo essencial. O conhecimento dessas estruturas e seu posicionamento no corpo do vegetal, aliados ao estudo químico, são resultados importantes do ponto de vista prático, pois indicam quais espécies e órgãos são mais promissores para futuras investigações.

Além dos óleos essenciais, as espécies de *Aldama* são conhecidas por produzirem as lactonas sesquiterpênicas, principalmente nos tricomas capitados glandulares, estruturas identificadas nesse estudo para folhas e caules das espécies *Aldama discolor* e *A. grandiflora*. Apesar de morfologicamente bastante estudados em outros representantes da família, os aspectos genéticos envolvidos com a biossíntese de compostos nesses tricomas foram pouco explorados até o momento. Nesse contexto, os dados obtidos sobre a ontogênese e diferenciação desses tricomas em *A. discolor*, bem como o entendimento do processo de acúmulo e secreção e da regulação gênica nos mesmos, trazem resultados inovadores para espécies nativas brasileiras.

ANEXOS

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DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Tese de Doutorado, intitulada "***Anatomia e fitoquímica de espécies de Aldama La Llave (Heliantheae - Asteraceae)***", desenvolvida no Programa de Pós-Graduação em Biologia Vegetal do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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Data: 20 de junho de 2016

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Declaração

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