Botânica Aplicada: metabólitos secundários na interação planta-ambiente

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**BOTÂNICA APLICADA: METABÓLITOS SECUNDÁRIOS NA
INTERAÇÃO PLANTA-AMBIENTE**

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CONTEXTO

A busca pelo entendimento dos fenômenos naturais de maneira ampla e contextualizada tem reforçado a necessidade do tratamento das questões científicas de modo multidisciplinar, tanto nas pesquisas como no ensino. Nesse contexto, a **Fitoquímica**, ciência resultante da união de duas grandes áreas: Biologia (*mais especificamente a Botânica*) e Química, contempla inerentemente essa característica. Nela, a composição química das plantas, principalmente daquelas substâncias chamadas de metabólitos secundários, é avaliada com diversos fins e distintos enfoques, muitas vezes associados à formação do profissional que conduz a pesquisa. Numa investigação conduzida por um profissional de formação biológica, entender o papel daquelas substâncias para a planta é muitas vezes o que rege sua pesquisa. Já para um químico a caracterização da estrutura daquela molécula e da sua via de síntese podem ser pontos mais instigantes naquele estudo. De qualquer maneira, independente da formação de cada profissional, o que se deve buscar é a sobreposição dessas duas grandes áreas na geração de conhecimento.

O estudo de extratos ou substâncias isoladas de espécies vegetais nativas pode revelar potenciais aplicações destas espécies de diversas formas, como, por exemplo, em suplementos alimentares, na indústria de cosméticos, como inseticidas naturais, ou mesmo proporcionar a descoberta e caracterização de uma nova molécula. Dentro da Botânica, estas investigações podem fazer parte de uma subárea da que chamamos **Botânica Aplicada**.

Dentro dessa linha de investigação, a procura de novas drogas para o tratamento de doenças como câncer, malária, leishmaniose, Alzheimer, entre outras, tem despertado grande interesse no conhecimento dos metabólitos secundários de inúmeras espécies de plantas. A necessidade de suplantar defesas adquiridas por muitos microrganismos contra drogas tradicionalmente usadas, também move essa busca por novos extratos e/ou substâncias ativas.

O reconhecimento do papel desses metabólitos na interação das plantas com outros organismos também estimula novos estudos. Aqui, a importância se dá não somente pelos benefícios ao ser humano, como, por exemplo, na descoberta de substâncias tóxicas a uma determinada praga de uma cultura, mas também, pela possibilidade de entendimento dos processos naturais que envolvem estes organismos, levantando questões como: *O que acontece com uma planta quando é exposta a um patógeno e/ou a um herbívoro? Porque alguns herbívoros se alimentam de uma planta e não de outra? O ataque de um patógeno/herbívoro a uma planta influencia a susceptibilidade de outras plantas naquele ambiente?*

Além disso, vivenciamos hoje um momento de grandes alterações climáticas. Nesse contexto, entender como as plantas se comportarão nesse novo cenário de aumento de poluentes atmosféricos, aumento nos teores de dióxido de carbono, aumento de

temperatura e consequentes restrições hídricas em algumas regiões, também reforça o interesse por esse aspecto aplicado da Botânica envolvendo o metabolismo secundário. *É possível observar alguma alteração nesses metabólitos em plantas submetidas a essas condições ambientais desfavoráveis (estressantes)? Se sim, essas alterações afetam o desempenho da planta? O conteúdo dessas substâncias pode ser manipulado na busca de variedades mais resistentes?*

Neste contexto, sem a pretensão de abordar todos os aspectos ligados à Botânica Aplicada dentro da Fitoquímica, este texto apresentará alguns estudos realizados por nossa equipe de pesquisa, nos quais são avaliados o papel dos metabólitos secundários na relação das plantas com fatores externos bióticos e abióticos visando contribuir na construção de respostas a algumas das questões acima.

OBJETIVO

O objetivo principal deste texto é demonstrar a importância do estudo dos metabólitos secundários visando enriquecer o conhecimento produzido em Botânica Aplicada. Para isso, serão descritas as principais classes de substâncias que compõem esse grande conjunto juntamente com suas vias de síntese. Além disso, explorando resultados obtidos ao longo dos anos, será retratado o papel dessas substâncias na interação das plantas com fatores bióticos, abióticos e suas aplicações.

BOTÂNICA APLICADA: METABÓLITOS SECUNDÁRIOS NA INTERAÇÃO PLANTA-AMBIENTE

METABÓLITOS SECUNDÁRIOS

As plantas, devido à sua forma sésil, diferem da maioria dos animais, pela ausência de movimentos, não podendo desta forma se deslocar quando estão submetidas a situações menos favoráveis ou estressantes. Assim, ao longo da sua história evolutiva os vegetais foram selecionados por outras estratégias de defesa. Muitos autores ressaltam que, uma das maneiras desses organismos lidarem com essas situações de estresse é através de substâncias que possibilitem, de alguma maneira, suplantarem os desafios. Dentre essas substâncias estão os metabólitos secundários.

Durante muito tempo, acreditou-se que essas substâncias eram produzidas sem uma função específica, caracterizadas como produtos sem valor, ou mesmo como resultado de algum erro metabólico, servindo como uma forma de desintoxicação das plantas (Taiz & Zeiger 2009). Entretanto, a partir da década de 1950, com o aumento do conhecimento e a descoberta cada vez maior de novos metabólitos, ficou claro o papel essencial dessas substâncias para a vida das plantas. Assim, esses metabólitos podem ser definidos como substâncias que não participam dos processos de formação de protoplasto e geração de

energia, muitos são mediadores em processos de interação das plantas com o ambiente, não apresentam ocorrência universal e exibem ampla diversidade estrutural (Dey & Harborne 1997), nem sempre são produzidas, podendo ser sintetizadas somente em resposta a estímulos especiais e, em muitos casos, suas funções não são completamente esclarecidas (Dewick 2009).

Através das vias do acetato-mevalonato (ou do ácido mevalônico) e do metileritritol fosfato (MEP) são produzidas as substâncias referidas como terpenos, ou terpenoides. Essas substâncias são formadas pela união de unidades pentacarbonadas (C5) chamadas isopreno, sendo agrupadas de acordo com o número dessas unidades na molécula: hemiterpenoides (C5), monoterpenoides (C10), sesquiterpenoides (C15), diterpenoides (C20), triterpenoides

(C30) e carotenoides (C40). Na via do acetato-mevalonato, localizada no citosol, há junção de três moléculas de acetil-CoA para a formação do ácido mevalônico, este é fosforilado, descarboxilado e desidratado para produzir o isopentenil difosfato (IPP), ou seu isômero - dimetilalil difosfato - DMAPP, que são as unidades básicas dos terpenos. Já, na via do MEP, que está localizada nos plastídeos, o IPP é formado após a união do gliceraldeído-3-fosfato e dois átomos de carbono derivados do piruvato; essa molécula passa por alguns rearranjos, formando um intermediário que é convertido em IPP. O isopentenil difosfato e o dimetilalil difosfato são as unidades pentacarbonadas ativas na biossíntese dos terpenos que se unem para formar as moléculas maiores (Taiz & Zeiger, 2009).

Os ácidos graxos, formados pela via do acetato-malonato (ácido malônico), tomam parte de diversas classes de substâncias denominadas genericamente de lipídeos. Essas substâncias – ácidos graxos – evidenciam a dificuldade, em alguns casos, da clara distinção entre metabólitos primários e secundários. Segundo Ohlrogge & Browse (1995) a via de síntese de ácidos graxos, em si, é parte do metabolismo primário das plantas, visto ser essencial ao crescimento do organismo.

O início da síntese dos ácidos graxos em plantas ocorre nos plastídeos através da condensação de uma molécula de acetil-CoA e uma de malonil-CoA que, após algumas reações, forma uma molécula com quatro átomos de carbono, que é alongada por condensações sucessivas de unidades de dois carbonos provenientes de novas moléculas de malonil-CoA até a formação dos ácidos graxos mais abundantes com cadeias carbônicas de 16 ou 18 átomos. Estes ácidos graxos são exportados para o retículo endoplasmático como ácidos graxos-CoA e são destinados à síntese dos lipídeos de membrana (glicerolipídeos), de reserva (triacilgliceróis) ou aqueles que formam a cutícula, por exemplo. Assim, os ácidos graxos quase nunca são encontrados livres nas células (Ohlrogge & Browse 1995). Neste texto, trataremos adiante um pouco mais em detalhes dos componentes da cutícula.

Através da via do acetato-malonato também são formados metabólitos como poliacetilenos e algumas substâncias aromáticas. Bem no início da via biossintética, a ação de complexos enzimáticos diferentes (policetídeo sintases) propicia o alongamento da cadeia resultando na formação de substâncias lineares de cadeias longas insaturadas e/ou hidroxiladas (poliacetilenos), que podem ciclizar e originar algumas substâncias aromáticas. As prostaglandinas, substâncias presentes em praticamente todos os tecidos de mamíferos em baixas concentrações, são sintetizadas a partir do ácido araquidônico (ácido graxo com 20 átomos de carbono). A importância dessas substâncias ao homem reforça ainda mais a necessidade da ingestão dos ditos ácidos graxos essenciais (ácido linoleico, ácido linolênico) obtidos dos vegetais na alimentação, precursores do ácido araquidônico (Dewick 2009).

Os compostos fenólicos, por sua vez, são caracterizados por possuírem pelo menos um anel aromático, no qual pelo menos um hidrogênio é substituído por uma hidroxila. Nas

plantas vasculares, cerca de 40% dos compostos fenólicos provêm da via do acetato-malonato e 60% são originados da via do ácido chiquímico, sendo esta, ausente em animais. O início da via se dá pela união do fosfoenolpiruvato com a eritrose 4-fosfato (produtos da fotossíntese) que por meio de algumas etapas, resulta na formação da primeira substância carbocíclica da via - ácido 3-dehidroquinico – precursor do ácido chiquímico. Antes, porém, da síntese do ácido chiquímico, há duas ramificações importantes nessa via: uma levando à síntese do ácido gálico, importante na formação dos galotaninos e elagitaninos e outra, a formação do ácido quínico, precursor de alcaloides como a quinina. Com a incorporação de outra molécula de fosfoenolpiruvato ao ácido chiquímico há a formação do ácido corísmico, a partir do qual são formados os fenólicos simples (ou ácidos benzóicos simples – C₆C₁), além da síntese dos aminoácidos aromáticos – triptofano, muito importante para síntese dos alcalóides indólicos, fenilalanina e tirosina. A partir da fenilalanina, principalmente, há formação dos fenilpropanoides (C₆C₃), que além de estarem presentes nas plantas com várias funções, levam a formação dos monômeros formadores da lignina, das lignanas e neolignanas, e cumarinas. Metabólitos importantes como flavonoides e estilbenos são formados pela junção desses fenilpropanoides (ácido *p*-cumárico) com moléculas de malonil-CoA (via acetato-malonato). As catequinas, um tipo de flavonoide, são as unidades formadoras dos taninos condensados, um importante polifenol (Dewick 2009, Taiz & Zeiger, 2009).

Muitos dos metabólitos secundários presentes nas plantas são substâncias nitrogenadas, biossintetizados a partir de aminoácidos. Entre eles estão inclusos os alcaloides, os glicosídeos cianogênicos e os glicosinolatos (Taiz & Zeiger, 2009). Os alcaloides são substâncias nitrogenadas de baixo peso molecular, com um ou mais átomos de nitrogênio dispostos como aminas primárias, secundárias ou terciárias, conferindo a essas substâncias, um caráter básico. Em meados do século passado, experimentos com precursores marcados revelaram que os alcaloides são biossintetizados a partir de poucos aminoácidos, sendo os principais ornitina, lisina, ácido nicotínico, tirosina, triptofano, ácido antranílico e histina, podendo haver incorporação às estruturas de porções provenientes de outras vias como acetato-malonato, chiquimato e acetato-mevalonato. Estas substâncias apresentam grande diversidade estrutural e sua classificação é baseada na natureza da porção que contém o nitrogênio, ou seja, na parte do esqueleto derivada dos aminoácidos. De acordo com Croteau et al. (2000) e Dewick (2009), são exemplos de alcaloides derivados de aminoácidos os pirrolidínicos, derivados da ornitina; os piperidínicos, derivados da lisina; quinolínicos, derivados do ácido antranílico; isoquinolínicos, derivados da tirosina e os indólicos, derivados do triptofano.

Os glicosídeos cianogênicos e os glicosinolatos, duas outras classes de metabólitos nitrogenados, também estão envolvidos em processos de defesa das plantas. No caso dos

glicosídeos cianogênicos, os principais aminoácidos precursores da sua síntese são fenilalanina, tirosina, vanilina, isoleucina e leucina. Quando tecidos de espécies que possuem glicosídeos cianogênicos são lesados, esses glicosídeos são hidrolizados por enzimas específicas também presentes nas plantas, havendo a liberação do ácido cianídrico (HCN), que atua impedindo a ação de enzimas envolvidas no processo de respiração celular, além de atribuir caráter tóxico às espécies que possuem essas substâncias. Os glicosinolatos, também conhecidos como óleo de mostarda, também são biossintetizados a partir de aminoácidos (tirosina, fenilalanina e triptofano, principalmente), mas, no entanto são também sulfatados. Da mesma forma que os glicosídeos cianogênicos, os glicosinolatos conferem toxicidade à planta que o possui somente se os tecidos forem lesionados, colocando em contato o glicosídeo com enzimas conhecidas como mirosinases. Nessa reação, são liberadas substâncias com odor característico, pungentes, incluindo isotiocianatos e nitrilas, como reportado por Croteau et al. (2000), Dewick (2009) e Taiz & Zeiger (2009).

INTERAÇÃO PLANTA-FATORES ABIÓTICOS

Os estudos com metabólitos secundários propiciaram grande avanço no desenvolvimento de técnicas de cromatografia e identificação de substâncias. Desde o século XIX, químicos orgânicos dedicam-se com empenho na elucidação de novas estruturas, aprimorando inclusive metodologias para síntese dessas substâncias, ou derivados, potencializando os efeitos de algumas substâncias ou mesmo, produzindo-as em larga escala. A descoberta da diversidade estrutural e, conseqüentemente, de possíveis aplicações destes metabólitos movem grande parte dos estudos realizados atualmente visando suas aplicações na indústria de cosméticos, perfumaria, alimentos, entre outras. O reconhecimento das propriedades biológicas de muitos desses metabólitos, resulta em um elevado número de pesquisas voltadas a busca por novas substâncias com atividades antimicrobiana (Girardi et al. 2014), antiproliferativa (Motta et al. 2011, Motta et al. 2013, Savietto et al. 2013), inseticida/acaricida (Myiashira et al. 2012; Righi et al. 2013) ou herbicida (Rial et al. 2014).

Além disso, os metabólitos secundários ganharam e vêm ganhando, destaque com as descobertas de suas múltiplas funções nas plantas, como regulação do crescimento e sustentação estrutural, interação com o ambiente, principalmente no que se refere à tolerância a temperaturas extremas ou estresse hídrico, além de seu papel com outros organismos no que tange a atração de polinizadores, dissuasores alimentares e defesas contra herbívoros e patógenos.

Diversos autores sugerem que a enorme variedade de pressões seletivas enfrentadas pelas plantas ao longo da sua história deve ter influenciado na vasta diversidade de metabólitos produzidos por esses organismos, os quais, de alguma maneira foram vantajosos às plantas propiciando benefícios evolutivos e sua sobrevivência (Wink 2003).

Com isso, ao longo do tempo, inúmeras teorias foram propostas tentando prever quais os tipos de metabólitos secundários são mais prováveis de serem sintetizados por uma determinada espécie, dependendo das pressões enfrentadas por ela, incluindo fatores ambientais e inimigos naturais (p. ex. herbívoros, patógenos).

A primeira teoria voltada a explicar os padrões observados de relações entre plantas e herbívoros foi a Teoria da Co-Evolução proposta por Ehrlich & Raven (1964). Esta, em essência, previa que as plantas produtoras de substâncias tóxicas a herbívoros, foram favoravelmente selecionadas, ocupando de forma vantajosa um novo nicho. Alguns desses herbívoros, por sua vez, ao longo da evolução foram selecionados de forma a contrapor essas “defesas”, colocando-os em vantagens em relação aos demais. Com essa “guerra-armamentista” de defesa/contraposição esses organismos, e suas relações, foram se diferenciando ao longo da história.

Essa teoria, entretanto, foi alvo de muita crítica nos anos seguintes, por envolver somente dois níveis tróficos nas suas explicações (plantas e herbívoros) e, principalmente, não levar em consideração os fatores ambientais. A partir de então, várias outras teorias foram sendo propostas incorporando, nas suas formulações, aspectos inerentes à planta e aos seus inimigos (herbívoros/patógenos), características do ambiente em que esses organismos eram encontrados (ricos ou pobres em nutrientes, ensolarados/sombreados, com disponibilidade hídrica ou secos), custo/benefício da produção de um metabólito quando a planta está ou não submetida às condições estressantes. No entanto, a ideia de uma estreita relação entre os metabólitos secundários produzidos pelas plantas e fatores externos (bióticos e abióticos) é amplamente aceita e oferece um campo fértil de possibilidades para investigações de diversas naturezas.

Nas linhas a seguir, serão discutidos aspectos do envolvimento de metabólitos secundários em interações das plantas com o ambiente, ressaltando alguns dos estudos por mim realizados em colaboração com pesquisadores e/ou alunos de graduação e pós-graduação.

No final da década de 1980, ainda na graduação, iniciei meu primeiro estudo envolvendo a análise dos metabólitos secundários das plantas em relação ao ambiente, investigando o papel das ceras foliares epicuticulares em espécies proximamente relacionadas, coletadas em áreas de cerrado e de mata atlântica (Varanda & Santos 1996 - Anexo 1).

O surgimento da cutícula foi uma novidade evolutiva extremamente importante para a conquista do ambiente terrestre pelas plantas (Simpson 2010). Essa camada lipofílica que recobre os tecidos aéreos de crescimento primário das plantas terrestres oferece, entre outras possibilidades, uma barreira contra perda de água por transpiração não estomática, controle da entrada e saída de solutos polares e também das trocas gasosas e vapores,

quando os estômatos estão fechados além de atenuar a incidência de radiação ultravioleta sobre os tecidos (Riederer 2006). Trata-se de uma camada formada por cutina, que consiste de uma rede de poliésteres derivados de ácidos graxos com cadeias carbônicas de 16 e 18 átomos hidroxilados, dihidroxilados e epóxi-hidroxilados, associada a ceras que podem estar entremeadas a essa rede (ceras cuticulares) ou acima dessa camada (ceras epicuticulares). Essas ceras são misturas complexas de séries de homólogos alifáticos de cadeia longa como alcanos, alcoóis e aldeídos formados por reações de redução destes ácidos graxos. De acordo com Riederer (2006) e Pollard et al. (2008), a cutícula também apresenta uma porção não hidrolizável denominada de cutano, derivada de ácidos graxos insaturados.

A biossíntese das ceras cuticulares envolve vários passos: síntese dos ácidos graxos C16:0/C18:0 nos plastídios, transferências desses ácidos graxos para o retículo endoplasmático, alongamento das cadeias carbônicas desses ácidos graxos até C26 – C32 através de complexos de elongases associadas ao retículo endoplasmático e, por fim, modificações nas cadeias carbônicas que levam a síntese dos componentes alifáticos presentes nessas ceras. Na maioria das plantas existem duas vias principais de síntese desses componentes: a via da redução acil que leva a formação dos álcoois primários e ésteres e a via da descarbonilação, responsável pela síntese dos aldeídos, alcanos, álcoois secundários e cetonas (Kunst & Samuel 2003).

Como uma das funções primárias associadas às ceras está relacionada ao controle de perda de água por transpiração não estomática, investigações visando correlacionar a espessura e/ou composição da cutícula e das ceras a características do ambiente sempre trazem informações interessante. Oliveira & Salatino (2000) estudando oito espécies do cerrado e da caatinga detectaram altos teores de cera (acima de $60\mu\text{g}\cdot\text{cm}^{-2}$) em seis delas, sendo as de caatinga todas desse grupo. Antes disso, um estudo feito no Laboratório de Fitoquímica com espécies de cerrado já havia relatado altos teores de cera nas folhas de várias espécies (Amaral et al. 1985).

Assim, o objetivo principal desse primeiro trabalho foi verificar a existência de variação nos teores de ceras foliares e na composição de alcanos entre espécies congêneres da mata e do cerrado.

O cerrado é um bioma que apresenta ampla variedade de tipos de fisionômicos, desde formas savânicas (campo limpo, campo sujo) até cerrado *sensu stricto* e cerradão. A aparência tortuosa das árvores e o solo seco conduziram muitos autores a sugerir a água como o fator limitante para a distribuição das espécies nesse bioma. No entanto, hoje se sabe que as características do solo são aquelas de maior importância. Além da deficiência de vários minerais, há nos solos do cerrado altos teores de alumínio (Al) que pode ser tóxico para muitas plantas, além da vegetação estar sujeita a queimadas periódicas (Coutinho 1982). A Mata Atlântica, assim como Cerrado, não é homogênea, apresentando formações variadas

abrangendo florestas úmidas, matas de araucária e florestas costeiras. Este bioma apresenta um alto índice pluviométrico e normalmente apresenta inverno seco e verão chuvoso. Ao lado de outros biomas, a Mata Atlântica e o Cerrado são considerados importantes *hotspots* de biodiversidade do planeta (Myers et al. 2000).

Nossa hipótese era que as espécies de cerrado, por estarem submetidas a condições ambientais mais estressantes de solo pobre e queimadas periódicas, apresentariam teores maiores de cera e hidrocarbonetos (alcanos) de cadeia mais longa. Entretanto, nem os resultados para teores de cera nem a análise mais detalhada dos alcanos confirmou o esperado; ainda que em algumas espécies do cerrado os teores de cera tenham sido maior, essa não foi a regra.

Numa leitura crítica atualizada, a análise da presença de terpenoides nessas ceras, além dos alcanos, teria sido bastante interessante. Como a incidência de herbivoria em espécies de cerrado é bastante expressiva, esses terpenoides, se presentes, poderiam ser considerados como uma primeira barreira anti-herbivórica para aquelas espécies. Além disso, atualmente, não teria a expectativa de alcanos de cadeias mais longas nas espécies de cerrado. Dentre os componentes das ceras, os alcanos são os mais eficientes como barreira contra perda de água (Oliveira et al. 2003). No entanto, água não é um fator limitante nesse bioma. Oliveira e colaboradores (2003) comparando espécies de cerrado e caatinga notaram diferenças expressivas nas espécies deste último ambiente, onde indubitavelmente há deficiência hídrica. Bourdenx et al. (2011) estudando mutantes de *Arabidopsis* com um gene envolvido na via de síntese de alcanos superexpressado, verificaram alterações na camada de cera e na síntese dos alcanos. Além disso, os autores observaram redução na permeabilidade da cutícula, acompanhada de menor susceptibilidade da planta à redução de disponibilidade hídrica do solo.

Por algum tempo, meu envolvimento com interpretações dos metabólitos secundários, especialmente componentes das ceras cuticulares, relacionados ao seu papel na interação com fatores bióticos e abióticos ficaram adormecidos.

Em 2006, entretanto, como parte do estágio de pós-doutorado realizado na Michigan State University (Michigan - EUA), retomei meus estudos com lipídeos de superfície, analisando componentes da cutina e das ceras de plantas selvagens de *Arabidopsis* submetidas à marcação por ^{14}C (Figura 2a). Neste trabalho, a taxa de incorporação do ^{14}C nos lipídeos solúveis (exceto ceras), na cera, na cutina e cutano foi medida ao longo de uma semana. A proporção da radioatividade nas ceras e na cutina, diferentes do observado para os glicerolipídeos, aumentou ao longo de todo experimento (Figura 2b). Em outras palavras, logo nas primeiras horas de exposição do ^{14}C , os lipídeos constituintes de membrana das células já estavam marcados, no entanto, a incorporação do ^{14}C nos lipídeos cuticulares foi mais lenta. A velocidade de incorporação do ^{14}C nos componentes da cutina foi ainda mais

Figura 2. Análise dos lipídeos de superfície em plantas selvagem de *Arabidopsis thaliana* crescidas em atmosfera com ^{14}C . A. Montagem do experimento de marcação. B. Medida da leitura de radioatividade nos diferentes componentes das ceras cuticulares extraídas das hastes das inflorescências (ALK – alcanos, KET – cetona, ALD – aldeídos, S-OH – alcoóis secundários, FFA – ácidos graxos livres, P-OH – alcoóis primários). C. Hipótese da existência de um *pool* de ácidos graxos pré-existent nas folhas que sirvam de precursores para síntese dos lipídeos da cutícula (linha pontilhada) (C16 – ácido graxo de cadeia carbônica com 16 átomos de carbono – ácido palmítico; C18 – ácido graxo de cadeia carbônica com 18 átomos de carbono – ácido esteárico; C26 – C32 – componentes com cadeias carbônicas de 26 a 32 átomos de carbono; FA – ácidos graxos, TAG – triacilglicerol). Dados apresentados no 17th International Symposium of Plant Lipids 2006.

lenta que os da cera. Essas observações nos induziram a sugerir que os ácidos graxos precursores para esses lipídeos de superfície podem não serem supridos diretamente dos plastídeos, mas sim, provenientes de um *pool*/pré-existente de ácidos graxos na célula, talvez triacilgliceróis (TAG) (Figura 2c). A presença de TAG em folhas, como lipídeos de reserva não é comum como nas sementes. No entanto, alguns autores como Chapman et al. (2013) têm demonstrado seu papel como uma reserva transiente. Esses resultados foram divulgados em um congresso internacional (Santos et al. 2006 - Anexo 2).

Estudos sobre a composição, controle da síntese e transporte para o exterior dos lipídeos de superfície são alvo de alguns grandes grupos de pesquisa dos Estados Unidos (Michigan State University) e Canadá (The University of British Columbia), envolvendo principalmente *Arabidopsis* e seus diversos mutantes disponíveis. Dessa maneira, acredito que a melhor forma de contribuição das pesquisas nesse tópico – lipídeos de superfície – seja aproveitar a biodiversidade disponível no país e as diferentes possibilidades climáticas para avaliação de plantas nativas e/ou cultivadas.

Nesse cenário, em decorrência da retomada com os estudos de cera, atualmente temos um projeto em andamento com variedades cultivadas de café diferencialmente resistentes à seca.

O café é uma das principais commodities agrícolas do mundo, sendo comercializado nas principais bolsas de mercadorias e futuros (Hein & Gatzweiler 2006). Segundo Deconto & Girardi (2008) o café arábica é uma das culturas que claramente necessitarão de uma reconfiguração geográfica na sua produção com o panorama de mudanças climáticas que vem se desenhando nos últimos anos. Para muitos estudiosos, haverá a necessidade de expansão do cultivo em áreas mais áridas, visto ser o aumento dessas áreas uma das possíveis consequências do aquecimento global. Por isso, há interesse na busca por plantas com maior resistência a condições de falta de água.

Com relação à resistência dessas plantas às condições de seca, Almeida et al. (2007) analisando plantas de 21 genótipos de *Coffea arabica* propuseram três grupos: tolerantes, intermediárias e susceptíveis ao processo de restrição hídrica. Estudos relacionando a deposição e composição das ceras nesses genótipos são praticamente inexistentes. Awati et al. (2012) analisando dois outros cultivares de arábica e um de robusta, verificaram diversas alterações em parâmetros fisiológicos relacionados às respostas a períodos de restrição hídrica, incluindo aumento da camada de cera em resposta ao estresse hídrico em um dos cultivares. A avaliação da composição da cera de algumas espécies de café aparece descrita em um artigo de Kitagami et al. (2013), enquanto a análise da influência da aplicação de fungicidas sobre a composição e morfologia da cera em folhas de café, foi alvo de outra investigação realizada por Lichston & Godoy (2006).

Nesse contexto, com o início desse projeto, avaliamos a composição da cera foliar cuticular de cerca de 10 indivíduos adultos de seis genótipos de *Coffea arabica*, distribuídos naqueles grupos descritos por Almeida et al. (2007), cultivados nos campos experimentais do IAC - Campinas. Os resultados foram apresentados no 21st International Symposium of Plant Lipids – Guelph (Canadá) em julho de 2014 (Santos et al. 2014 - Anexo 3) e o manuscrito está em preparação.

As ceras foliares foram extraídas por imersão em diclorometano, derivatizadas com BSTFA (N,O-Bis(trimetilsilil)trifluoroacetamida) e analisadas através de cromatografia gasosa acoplada a espectrometria de massas. Apesar dos dados prévios apontarem alcanos, alcoóis primários e ácidos graxos livres como componentes principais para esta espécie (Kitagami et al. 2013), em todas as nossas amostras, os triterpenos pentacíclicos foram os componentes majoritários, perfazendo de 40 a 60% de toda cera. Foram detectados cinco triterpenos, dois dos quais ainda não foram completamente identificados, sendo os demais uvaol, ácido oleanólico e ácido ursólico; este último correspondendo, em média, a cerca de 30% do total de cera da folha. Os homólogos C29 e C31 foram os alcanos principais; C30 e C32 foram os principais alcoóis primários detectados. Ácidos graxos livre de cadeia longa foram detectados somente em quantidade traço (abaixo de 1%). Cafeína é, sabidamente, um dos principais componentes das folhas de café. No entanto, a presença de alcaloides em ceras cuticulares não é comum. Até o momento, a presença dessa substância em ceras foi descrita somente para *Ilex paraguariensis* (Aquifoliaceae), espécie nativa da América do Sul, muito usada na preparação do mate, típico das regiões do sul do Brasil (Athayde et al. 2000). A presença desse alcaloide foi ubíqua nas ceras de todos os genótipos analisados no nosso trabalho. Entretanto, ainda que com vários resultados interessantes, não foi observada qualquer correlação entre o teor de ceras cuticulares e/ou dos seus componentes com a prévia classificação dessas plantas nos grupos propostos por Almeida et al. (2007) (Figura 3).

Apesar do reconhecido papel da camada cuticular como barreira a perda de água pelas plantas, essa característica não é a única apresentada pelos vegetais. Movimento das folhas, estômatos protegidos em depressões da epiderme, regulação eficiente do movimento dos estômatos, redução no tamanho das folhas e alterações em nível celular através do ajuste osmótico são também alguns mecanismos apresentados pelas plantas.

Assim, analisando os resultados obtidos com os genótipos de café, a falta de correlação entre os teores de cera das folhas com a caracterização dos genótipos é justificada por serem amostras de plantas adultas, já estabelecidas em campo e adaptadas às condições ambientais existentes. A questão que fica é: *Será que plantas jovens desses genótipos, responderiam da mesma forma se submetidas a períodos de restrição hídrica?* Segundo Shepherd et al. (2006) a deposição de cera é uma resposta ao estresse ocasionado pela falta de água, podendo esse mecanismo de defesa ocorrer rapidamente dentro de

poucos dias. Cameron et al. (2006) observaram um nítido aumento na camada cerosa, acompanhado de uma menor velocidade na perda de água por evapotranspiração em plantas jovens de tabaco submetidas a restrições hídricas.

Figura 3. Análise das ceras foliares cuticulares de genótipos de *Coffea arabica* com diferentes níveis de resistência a seca. A. Teores de ceras cuticulares totais em µg.cm⁻² (genótipos tolerantes: Laurina e Semperflorens; genótipos intermediários: Mundo Novo, Catuaí, Caturra Vermelho; genótipo susceptível: Bourbon Vermelho). B. Porcentagem das principais classes de componentes da cera no genótipo Bourbon Vermelho (HC – alcanos, FFA – ácidos graxos livres, PA – alcoóis primários, PTA – triterpenoides ácidos pentacíclicos). C. Estruturas de dois principais triterpenoides encontrados nas ceras cuticulares de *Coffea arabica*. Dados apresentados no 21st International Symposium of Plant Lipids 2014.

Dessa maneira, a análise da quantidade, composição e estrutura das ceras cuticulares de plantas jovens de dois genótipos de *Coffea arabica* (tolerante x susceptível a seca) será tema de um mestrado a ser iniciado ainda em 2015 sob minha orientação.

Além dos componentes das ceras e os efeitos da restrição hídrica, muitos outros metabólitos secundários podem estar envolvidos em processos de defesa das plantas a diversas outras alterações ambientais como, por exemplo, temperaturas muito baixas ou muito altas, diferenças na incidência de radiação, entre outros, e em função disto, muitas vezes essas plantas podem servir como bioindicadoras dessas alterações como, por exemplo, aumento de poluentes (Furlan et al. 2008, Furlan & Rezende 2009). Nesse contexto, colaborei em algumas análises de projetos desenvolvidos por outros pesquisadores do Laboratório de Fitoquímica, utilizando goiaba (*Psidium guajava* – Myrtaceae) como bioindicadoras de poluentes industriais (Furlan et al. 2006, Furlan et al. 2010).

As possibilidades de estudos relacionando plantas a fatores ambientais abióticos são inumeráveis e altamente diversificadas. A riqueza de possibilidades envolvendo os biomas que temos somente em nosso país já ilustra essa afirmação. Se incluirmos, nessa diversidade fisionômica, o ambiente marinho, nossas possibilidades ficam ainda mais ilimitadas.

Nesse contexto, cabe apresentar os resultados de nossas investigações recentes envolvendo espécies de algas vermelhas (Rhodophyta) submetidas a condições de alta intensidade luminosa. Esse projeto é fruto da inestimável colaboração estabelecida com a Dra Fungyi Chow do Laboratório de Algas Marinhas “Edson José de Paula” do Departamento de Botânica do IB, além da pós-graduanda Priscila Bezerra Torres.

No ambiente aquático, as algas vermelhas, assim como outros organismos, estão expostas a condições de excesso de luz ao longo dos dias, das estações do ano, do ciclo de marés ou como mudanças repentinas no tempo (Schubert 2001), gerando um estresse luminoso. Nestas circunstâncias, os cloroplastos são muito afetados, o excesso de energia pode sobrecarregar o aparato fotossintético, aumentando a produção de espécies reativas de oxigênio (EROs) (Müller et al. 2001). Essas EROs, apesar de serem produzidas em diversos processos biológicos nos organismos, quando em excesso podem ser altamente danosas ocasionando danos a proteínas, ácidos nucleicos, chegando a causar a morte do organismo. Assim, as algas vermelhas têm sido selecionadas com diversos mecanismos de fotoproteção que envolvem alterações morfológicas, anatômicas e fisiológicas (Simioni et al. 2014).

Em nossa investigação analisamos a taxa de crescimento e o comportamento *in vitro* de duas classes de pigmentos envolvidos na fotossíntese – clorofila a e carotenoides – em *Gracillariopsis tenuifrons* (C. J. Bird & E. C. Oliveira) Fredericq & Hommersand (Torres et al. 2014 - Anexo 4). Ápices de talos do gametófito feminino dessa alga foram cultivados, em laboratório, sob três intensidades luminosas: 60 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ (controle), 600 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ e

1.000 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ durante sete dias, sendo a taxa de crescimento, teor e composição dos pigmentos fotossintetizantes analisados diariamente.

Os resultados obtidos mostraram-se bastante interessante. Apesar da intensa mudança de cor, passando do vermelho para o amarelo, ter sido observado nos talos mantidos em alta intensidade luminosa, não foi observada queda na taxa de crescimento. Assim, podemos sugerir que, pelo menos para esta espécie, em laboratório, a perda de pigmentação, sugerida por outros autores como indicativo de queda na capacidade fotossintética e consequentemente na produção de biomassa, não está associada com um dano severo no aparato fotossintético. Ao contrário, a perda na coloração pode estar associada a uma estratégia de fotoproteção e fotoaclimação. Perda de coloração em algas vermelhas pode ser observada em campo em cenários de alta irradiância. Díes et al. (2012) sugerem que o aumento no número de espécies de algas com pigmentações atenuadas observadas em campo, pode ser consequência de alterações climáticas globais, tornando a compreensão dos mecanismos de fotoproteção desses organismos, muito importante do ponto de vista ecológico e também econômico.

Em relação aos pigmentos, clorofila a, β -caroteno e zeaxantina foram os principais. No grupo controle, não foram observadas diferenças significativas nestes três pigmentos ao longo dos sete dias. No entanto, para os grupos submetidos a alta intensidade, houve uma drástica redução nos níveis de clorofila a e β -caroteno, alcançando níveis entre 40% - 50% menores no sétimo dia quando comparado ao tempo zero. No caso da zeaxantina, foi observado um aumento nos primeiros dias, seguido de queda até o final do experimento muito menos significativa que nos casos anteriores. No entanto, apesar da queda na quantidade de zeaxantina no decorrer do experimento, as razões zeaxantina/ β -caroteno e zeaxantina/clorofila a foram sempre crescentes.

Considerando que a clorofila a e β -caroteno podem estar envolvidos na captura da energia luminosa no processo de fotossíntese, a queda desses pigmentos em condições de alta intensidade luminosa pode ser uma estratégia em *Gracillariopsis tenuifrons* para diminuir a absorção de energia, evitando as consequências danosas apontadas acima. No caso da zeaxantina, no entanto, o aumento contínuo das razões zeaxantina/ β -caroteno e zeaxantina/clorofila a demonstrou que a síntese desse pigmento foi muito maior nessa alga durante sua aclimação, podendo estar envolvido num processo de fotoproteção, ou seja, aparentemente esta alga lança mão de pelo menos duas estratégias diferentes para se proteger de uma situação de estresse, isso considerando somente os pigmentos fotossintetizantes.

Dessa forma, finalizo esta primeira parte deste texto esperando ter apresentado alguns aspectos importantes da avaliação dos metabólitos secundários em plantas em

resposta a fatores abióticos do ambiente. Estes estudos trazem além dos resultados acadêmicos, possibilidades de interpretações voltadas ao possível comportamento de uma espécie se submetida a um fator de estresse, seja esse artificial ou decorrente de um processo natural.

INTERAÇÃO PLANTA-FATORES BIÓTICOS

Além das investigações realizadas com a interação das plantas e fatores abióticos, sempre houve interesse em entender o papel desses metabólitos secundários nas interações com fatores bióticos. Para isso, iniciamos alguns estudos visando entender possíveis modificações nas plantas frente a infecção por patógenos, no caso vírus.

Os metabólitos secundários são reconhecidamente importantes na defesa contra patógenos. Nas infecções por fungos, por exemplo, diversos estudos demonstraram o aumento na concentração de compostos fenólicos (p.ex. Kröner et al. 2012). Quando o patógeno é um vírus, no entanto, o tipo de alteração nos metabólitos secundários é bastante variável, havendo aumento, diminuição ou nenhuma alteração, dependendo do sistema planta/patógeno estudado (Bruni et al. 2007; Duarte et al. 2008).

Os vírus são patógenos intracelulares obrigatórios, pois só completam seu ciclo de vida dentro da célula do hospedeiro. Os fitovírus, ou seja, vírus que infectam plantas possuem como material genético DNA ou RNA, sendo este último, a maioria deles. O genoma dos fitovírus está entre os menores dentre os genomas virais, codificando de uma a 12 proteínas responsáveis pela replicação, pela estrutura viral, pela seletividade na transmissão, pela supressão do sistema de defesa do hospedeiro e pelo movimento célula-célula do vírus. Os vírus são organismos que apresentam estratégias diferentes de replicação e interação com o hospedeiro, sendo esse, um dos motivos que podem explicar as diferenças observadas nos padrões de alteração nos metabólitos secundários das plantas, já que a interação do patógeno com seu hospedeiro é bastante variável (Hull 2009).

Os sintomas presentes nas plantas infectadas podem ser locais, ou seja, restritos ao local onde aconteceu a infecção viral, ou sistêmicos, aparecendo em diversos órgãos do vegetal. Este último é consequência da movimentação do vírus na planta, caracterizando uma infecção sistêmica (Hull 2009). Em algumas doenças virais, os sintomas sistêmicos podem acarretar diminuição do tamanho da planta infectada, padrões não uniformes de coloração das folhas, amarelecimento e enrolamento das folhas, aparecimento de necrose, etc, gerando grandes prejuízos econômicos.

Nesse contexto, iniciamos nossas investigações nessa linha de pesquisa, analisando as possíveis alterações no teor e na composição do óleo volátil de indivíduos de manjerição (*Ocimum basilicum* L. – Lamiaceae) desafiados artificialmente com um isolado de vírus obtido de plantas infectadas em campo (Nagai et al. 2011 – Anexo 5), trabalho que foi tema da IC de

Alice Nagai e contou com a colaboração dos Drs Alexandre Chaves e Lígia Duarte do Instituto Biológico de São Paulo.

O manjeriço é uma planta bastante importante economicamente, sendo cultivada em diversas regiões do mundo, principalmente, para obtenção dos óleos voláteis. Essa espécie é usada como matéria-prima para a indústria de fármacos e óleos, além de ser empregada também na culinária (Duman et al. 2010). Nossos resultados demonstraram que a infecção viral gerou alterações quantitativas e qualitativas no perfil do óleo volátil das folhas de manjeriço. Quantitativamente, a proporção dos principais componentes desse óleo (metil-eugenol e *p*-cresol,2,6-diterci-butílico) foi significativamente alterada. Em termos qualitativos o linalol e o eugenol foram detectados somente nas plantas sadias, enquanto o bergamoteno somente nas infectadas. Dessa forma, sugerimos que a infecção viral altera o metabolismo dessa espécie, levando a ativação/inativação de algumas etapas das vias biossintéticas desses componentes, propiciando alteração na composição do óleo volátil. Assim, fica evidente o papel do controle fitossanitário no transporte e estabelecimento de novas culturas por produtores rurais. A infecção por um patógeno pode, como demonstrado, alterar o metabolismo da planta resultando na modificação de suas propriedades biológicas e no seu valor econômico.

A obtenção desses resultados nos levou a analisar outros sistemas planta/vírus. Então, foram realizadas duas dissertações de mestrado avaliando a influência da infecção viral no perfil de compostos fenólicos dessas plantas. Em infecções de plantas por fungos, como dito anteriormente, há várias evidências do aumento na síntese dessas substâncias. No caso de infecções por vírus, os trabalhos existentes mostram possibilidades diversas.

Uma das dissertações foi desenvolvida pela pós-graduanda Alice Nagai, na qual o sistema estudado foi *Physalis angulata* L. (Solanaceae) infectada por um isolado do *Potato virus Y* (PVY⁰). Neste estudo, observamos, no geral, uma queda nos teores de fenóis totais e flavonoides totais nas plantas infectadas quando comparadas àquelas sadias. Entretanto, um ponto muito interessante foi notar que a injúria mecânica causada no processo da infecção levou a uma redução mais acentuada nesses metabólitos, quando comparada à presença do vírus. Em outras palavras, os valores observados nas plantas desafiadas artificialmente com o vírus, ou seja, aquelas que tiveram suas folhas friccionadas com a solução tampão contendo o inóculo viral, foram menores que os observados nas plantas controle (sem fricção), mas maiores que aqueles observados nas plantas friccionadas somente com tampão (Nagai et al. *submetido* – Anexo 6).

Em um estudo com cultivares de *Solanum tuberosum* L. (Solanaceae) tolerantes e resistentes ao PVY^{NTN}, Kreft et al. (1999) observaram que naqueles tolerantes a ausência de sintomas severos era acompanhada de queda de pelo menos um dos principais flavonoides foliares detectados naquela espécie. Já, nos resistentes, este mesmo flavonoide apresentava

leve aumento. Com análise mais detalhada dos flavonoides em *Physalis angulata*, pudemos sugerir que esta espécie deve ser tolerante ao PVY⁰, uma vez que não foram detectados sintomas severos nas plantas infectadas e todos os flavonoides detectados apresentaram queda.

Como já dito, a forma com que os vírus se replicam nas plantas hospedeiras é bastante variável, o que pode explicar em parte as diferenças nas alterações observadas nos metabólitos secundários dessas plantas infectadas. Essa diferença ficou muito evidente para nós com os resultados obtidos no segundo modelo utilizado para verificar a influência da infecção viral no metabolismo fenólico das plantas, tema da dissertação do mestrando Armando Toshikatsu Tomamitsu sob orientação do Dr Marcelo Eiras (Instituto Biológico de São Paulo) e sob minha co-orientação.

Analisamos o teor de fenóis totais e flavonoides totais em folhas de plantas do maracujazeiro (*Passiflora edulis* Sims. – Passifloraceae) desafiadas com o CABMV (*Cowpea aphid-borne mosaic virus*). Esse vírus causa uma doença conhecida como endurecimento do fruto, acarretando prejuízos econômicos enormes a essa cultura. Nessas plantas, para nossa surpresa, apesar dos sintomas muito evidentes, incluindo redução de 80% na altura das plantas infectadas, não foi observada diferença significativa nos teores de fenóis totais ou flavonoides (Tomomitsu et al. 2014 - Anexo 7).

Sabe-se que os mecanismos envolvidos nas respostas de defesa das plantas contra patógenos são extremamente complexos. Além de haver barreiras constitutivas da planta, há ativação de respostas imunes inatas local e/ou sistemicamente. Em linhas gerais, alguns autores propõem:

- a) Inicialmente, há o reconhecimento do eliciador do patógeno pelo receptor da planta;
- b) A partir daí, uma das primeiras respostas da planta é a explosão oxidativa, na qual há produção de espécies reativas de oxigênio (ERO), as quais possuem diversas funções, como a morte do patógeno, a reação de hipersensibilidade, ou a indução da SAR, que é a resistência sistêmica adquirida. Esta atua em órgãos distantes dos infectados, tornando-os imunes a infecções causadas pelo mesmo patógeno ou a patógenos muito relacionados;
- c) Além da explosão oxidativa, pode ocorrer alteração na concentração de compostos como as políminas e óxido nítrico de maneira muito rápida após a infecção; estas moléculas estão envolvidas na síntese de ácido jasmônico e ácido salicílico, também envolvido no desencadeamento da SAR;
- d) Como forma de eliminar as EROs produzidas em excesso no processo de estresse, há alteração do sistema antioxidante da planta que envolve o equilíbrio do ácido ascórbico e glutatona;

e) Os sinalizadores produzidos (NO, poliaminas) podem influenciar a síntese dos metabólitos secundários, ativando/desativando algumas etapas de síntese de substâncias fenólicas, por exemplo. Juntamente com o ácido ascórbico e a glutatona, os compostos fenólicos podem ajudar a planta a eliminar as espécies reativas de oxigênio.

Os resultados encontrados nos três sistemas planta/vírus investigados, principalmente nos dois últimos apresentados, geraram algumas questões que direcionam os próximos trabalhos já em andamento: *a) Como a infecção viral é percebida pela planta? b) Quais moléculas estão envolvidas nesse processo? c) Há diferença na velocidade e na forma com que plantas tolerantes e susceptíveis respondem a esse estresse? d) Será que diferenças no processo de percepção/sinalização da infecção explicam os padrões diferentes de alterações nos metabólitos secundários?*

Assim, atualmente trabalhamos, em uma tese de doutorado, com dois sistemas planta/vírus investigando o teor de sinalizadores (NO, poliaminas, ácido jasmônico e ácido salicílico), a ativação do sistema antioxidante da planta (ácido ascórbico e glutatona), além do metaboloma, com ênfase nos compostos fenólicos, dessas plantas saudáveis e infectadas.

APLICAÇÕES DOS METABÓLITOS DE PLANTA

O interesse na retomada dos estudos com café, apontado acima com relação às análises de lipídeos de superfície, surgiu na verdade de trabalhos anteriores com essa espécie e o uso de seus metabólitos. Dentro do interesse pela compreensão da atuação dos metabólitos secundários nos processos de interação da planta com fatores externos e somados a isso a Botânica Aplicada, desenvolvemos alguns estudos avaliando o papel dessas substâncias sobre as formigas cortadeiras. Vários estudos foram feitos no laboratório de Fitoquímica sendo, alguns deles envolvendo espécies de café.

A cafeína, importante metabólito presente no café, é um estimulante do Sistema Nervoso Central (SNC), com efeito inibidor do sono, restaurando a atenção e fornecendo uma “dose extra” de energia. Dados recentes mostram que os efeitos dessa substância em animais invertebrados e vertebrados são muito semelhantes em diversos aspectos, no entanto, detalhes relacionados aos mecanismos de ação desse alcaloide ainda precisam ser desvendados (Mustard 2014).

Mazzafera, em 1991, estudou a influência da cafeína no ataque de saúvas a cafeeiros, e sugeriu uma correlação negativa entre o teor dessa substância e os ataques à planta.

As saúvas, ou formigas cortadeiras do gênero *Atta*, são restritas ao continente americano com distribuição do centro da Argentina até o sul dos Estados Unidos. Esses insetos vivem em associação com o fungo simbiote *Leucoagaricus gongilophorus* (Möller) Singer de maneira tão estreita que é impossível a existência de um separado do outro. Nessa interação, a formiga coleta materiais vegetais frescos para o cultivo do fungo e este, por sua

vez, produz hifas com dilatações (gongilídios) que são ricas em glicogênio e podem ser prontamente assimiladas pelas larvas (Herrera & Pellmir 2002). Esse fungo também é a única fonte de alimento para a rainha.

Muitas vezes, esses insetos são considerados pragas por causarem grandes problemas a agricultura brasileira (Fernandes et al. 2002). Já dizia Auguste de Saint-Hilaire (1779-1853) - "Ou o Brasil acaba com a saúva ou a saúva acaba com o Brasil". Os grandes prejuízos causados se devem, principalmente, ao tamanho do saúveiro, o que demanda grande quantidade de folhas para o fungo simbiote (Oliveira 2006). Atualmente, o controle dessa praga é feito por meio de inseticidas sintéticos de amplo espectro de ação. Além da ação indiscriminada, outros efeitos como a contaminação ambiental, o excesso de resíduos nos alimentos, e a seleção de insetos resistentes são bastante indesejáveis nesse combate. Por isso, existe a busca por "inseticidas" naturais, obtidos de plantas, por exemplo, que sejam específicos às formigas cortadeiras, ao seu fungo simbiote ou a ambos (Fernandes et al. 2002) e que tenham baixa permanência no solo. No entanto, uma estratégia de controle eficiente em larga escala e menos danosa, ainda não foi alcançada (Sumida et al. 2010).

Nesse contexto, sabendo que a o teor de cafeína parece influenciar no forrageamento das saúvas, resolvemos avaliar se essa substância teria efeito sobre esse inseto, sobre o fungo simbiote ou ambos.

Como citado anteriormente, as formigas-cortadeiras e o fungo simbiote apresentam uma relação mutualística muito intrincada. Estudos apontando a dificuldade de estabelecer cultivos *in vitro* de fungos mutualistas já eram conhecidos, principalmente, pelo crescimento muito lento desses organismos (Ribeiro et al 1998, Loeck et al. 2004). Assim, nosso primeiro desafio foi separar estes dois organismos.

O cultivo *in vitro* do *Leucoagaricus gongylophorus* a partir de formigueiros mantidos no Laboratório de Fitoquímica foi desenvolvido no Centro de Pesquisa em Micologia do Instituto de Botânica de São Paulo com a colaboração da Dra Adriana Gugliotta por dois alunos de IC na época (Carlos Miyashira e Daniel Tanigushi). Neste estudo, foi feita a comparação da taxa de crescimento do fungo em dois meios de cultura (Miyashira et al. 2010 - Anexo 8).

As principais contribuições desse trabalho foram: a) descrição da forma de obtenção e transporte do fungo a partir do formigueiro no início do cultivo; b) definição do melhor meio de cultura sólido para o cultivo desse fungo, usando como parâmetros a melhor visibilidade para acompanhamento do crescimento do fungo em placa de Petri e a simplicidade na composição; e c) proposição de uma forma mais acurada de medir o crescimento do fungo, através da medida da expansão radial do inóculo inicial em quatro eixos previamente estabelecidos.

Com o método de cultivo do fungo bem definido, pudemos então testar o efeito da cafeína no fungo, impregnando este metabólito no meio de cultura, e na formiga. Para isso, avaliamos a sobrevivência de formigas operárias separadas do formigueiro e mantidas com o oferecimento de dietas sólidas (Bueno et al. 1997) incorporadas com diferentes concentrações de cafeína. Este trabalho foi o tema da dissertação de mestrado do biólogo Carlos Miyashira.

Foram avaliados os efeitos de quatro concentrações de cafeína (0,01%, 0,05%, 0,10% e 0,50%) tanto para os fungos como para as formigas. Três padrões de crescimento do fungo foram observados: a) crescimento igual ao controle (somente o meio de cultura) em placas com 0,01% de cafeína, b) redução intermediária no crescimento do fungo, observada desde o 14º dia do cultivo, em placas com 0,05% de cafeína e c) redução drástica do crescimento do fungo nas concentrações de 0,10% e 0,50%, sendo, nesta última, observada a morte do inóculo inicial já na primeira semana. Já, para as formigas, não houve diferença significativa no M50 (dia em que metade das formigas estavam vivas após o início do bioensaio) entre qualquer uma das concentrações de cafeína (Miyashira et al. 2012 – Anexo 9).

Com este estudo, sugerimos que a seleção diferenciada de coleta das folhas de espécies de *Coffea* pelas saúvas, como apontado por Mazzafera (1991), pode ser explicada pela sensibilidade do fungo a esse metabólito. O prejuízo no crescimento do fungo dentro do ninho deve servir como aviso às formigas de toxicidade daquela fonte de alimento.

Dessa forma, voltando ao tema central que é o uso dos metabólitos secundários dentro da Botânica Aplicada, uma possibilidade decorrente destes resultados seria o uso deste metabólito como fungicida. Iscas contendo cafeína, misturadas a alguma substância atrativa às formigas, poderiam ser posicionadas próximas às entradas dos formigueiros. Se carregadas pelas operárias e incorporadas ao jardim de fungos, a cafeína funcionaria como fungicida, fazendo controle dessas saúvas com menor impacto. Estudos de campo para verificar a eficiência e também o custo são necessários.

Ainda nessa linha de busca por substâncias úteis no controle das saúvas, realizamos alguns outros estudos testando plantas e métodos de aplicação diferentes.

Poucos estudos analisando a toxicidade de óleos de semente e suas frações contra formigas cortadeiras e seu fungo mutualístico já foram realizados. Fernandes et al. (2002) demonstraram a atividade de óleos extraídos de semente de *Citrus* sobre esses dois organismos. Toxicidade contra as saúvas também foi verificada com óleo de gergilim (*Sesamum indicum* L. – Pedaliaceae) e óleo de neem (*Azadirachta indica* A. Juus. – Meliaceae) (Morini et al. 2005, Santos-Oliveira et al. 2006).

Como tema de um projeto de IC - Emerson Alonso- avaliamos o potencial tóxico contra as saúvas de óleos de sementes de *Ricinus communis* L. (mamona) e *Jatropha curcas* L. (pinhão-mansão), duas espécies de Euphorbiaceae importantes economicamente

exatamente devido aos óleos das sementes. A toxicidade foi avaliada de duas formas: uma baseada no oferecimento de dietas artificiais incorporadas com concentrações diferentes desses óleos, como descrito para o ensaio com cafeína e a outra, através de bioensaio de contato, no qual uma gota de soluções de cada óleo foi aplicada no pronoto de cada formiga. Os resultados mostraram que ambos os óleos foram tóxicos para as saúvas nas duas formas de ensaios testadas, sendo o óleo de pinhão-manso mais efetivo por apresentar resultados significativos de M50 nas menores concentrações (Alonso & Santos 2013 – Anexo 10).

O diferencial nesse estudo, em relação aos demais, foi o monitoramento das visitas das formigas à dieta no bioensaio por 48h através de filmagem. Aqui o objetivo foi investigar uma possível atividade deterrente desses óleos para as formigas. Com esses dados, foi possível observar, como esperado, menor número de visitas às dietas com maior concentração do óleo de mamona. No caso do óleo de pinhão-manso, essa relação não foi tão evidente. Com isso, sugerimos que em baixas concentrações, as formigas não percebem a presença do óleo de pinhão-manso na dieta, alimentando-se dela normalmente. Essa observação nos levou a sugerir, no artigo, que esse óleo também poderia ser uma possibilidade de controle desses insetos se incorporado a iscas, como já mencionado acima para a cafeína, pois é importante lembrar que esse óleo foi eficiente na morte das formigas mesmo em baixa concentração.

Entretanto, nem sempre os ensaios em laboratório nos trazem as respostas desejadas. Em outro trabalho envolvendo esses ensaios com saúvas em laboratório, como parte da IC de Milena Timich, investigamos o papel de extratos foliares de uma espécie *Croton* nessa relação. *Croton* é um gênero de Euphorbiaceae com mais de 1200 espécies espalhadas pelo mundo em regiões tropicais e subtropicais (Govaerts et al. 2000), rico em componentes com atividade biológica. *Croton urucurana* Baill., a espécie analisada, é bastante conhecida na medicina popular por possuir efeito analgésico, além de ser utilizada no tratamento de reumatismo e câncer (Salatino et al. 2007). Além disso, Silva et al. (2009) observaram expressiva mortalidade em larvas de *Anagasta kuehniella* Zeller (Lepidoptera: Pyralidae) submetidas a algumas frações de extratos dessa planta, indicando um possível uso como inseticida natural.

Através do teste de contato direto, descrito acima, testamos concentrações diferentes de extratos foliares de diferentes polaridades e não detectamos efeito inseticida frente às formigas-cortadeiras com qualquer um deles. Ou seja, não houve diferença significativa entre o dia-médio em que 50% das formigas estavam mortas (M50) do controle comparado a qualquer M50 das formigas submetidas às diferentes concentrações dos extratos (Timich & Santos, *submetido* – Anexo 11). Nesse caso, o possível papel inseticida de *Croton urucurana* não pode ser confirmado para as saúvas.

CONSIDERAÇÕES FINAIS

Esse texto apresentou uma breve ideia das possibilidades de pesquisa dentro da Fitoquímica, voltadas a Botânica Aplicada utilizando os metabólitos secundários das plantas como ponto central da investigação.

A busca da compreensão do papel dessas substâncias nas relações das plantas com fatores externos tem permeado minha vida acadêmica desde muito cedo, ainda que desviada em alguns momentos por dedicação a outros aspectos também valiosos no estudo dos metabólitos secundários. Os artigos e comunicações em congressos utilizados na redação deste texto, além de contribuírem com o desenvolvimento do conhecimento científico, propiciam e impulsionam a continuidade das investigações que envolvem a mim e alguns dos meus alunos de graduação e pós-graduação. Atualmente, temos nos dedicado efetivamente em aprofundar nosso conhecimento nas repostas desencadeadas pelas plantas quando submetidas a condições de estresse, seja esse biótico ou abiótico.

Finalmente, mas não menos importante, é necessário ressaltar o papel dessas pesquisas como instrumento de ensino. Com os estudos envolvendo as saúvas, pudemos trabalhar com estudantes do Ensino Médio em projetos de pré-IC, propiciando o contato com a metodologia científica, o convívio com o ambiente universitário esperamos com isso, ter despertado o interesse pela Ciência. Ainda, num momento em que vislumbramos um ensino contextualizado, integrativo e transdisciplinar vejo que abordar a Botânica Aplicada, utilizando o viés dos metabólitos secundários traz uma oportunidade ímpar no entendimento, por exemplo, de como as plantas, das quais somos tão dependentes, puderam se adaptar a um mundo sujeito constantemente a alterações climáticas expressivas.

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ANEXOS

Anexo 1

Varanda, E.M., Santos, D.Y.A.C. 1996. Ceras foliares epicuticulares de espécies congêneres de Mata e de Cerrado. **Acta botânica brasilica** 10: 51-58.

CERAS FOLIARES EPICUTICULARES DE ESPÉCIES CONGÊNERES DA MATA E DO CERRADO¹

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RESUMO- (Ceras foliares epicuticulares de espécies congêneres da mata e do cerrado). Espécies de cerrado e mata foram analisadas quanto à sua composição em ceras foliares epicuticulares e de seus componentes hidrocarbonetos. Observou-se nas espécies de cerrado uma tendência a teores de ceras pouco maiores que os de espécies de mata estacional semidecídua. A porcentagem de hidrocarbonetos nas ceras foi maior na maioria das espécies de mata que nas espécies congêneres de cerrado. Pela análise em CG, os hidrocarbonetos mostraram predominância de C_{29} e C_{31} apresentando um comprimento médio da cadeia de carbono dos homólogos menos variável em espécies de mata, em torno de 30,5, que de cerrado nas quais este valor variou de 28,5 a 31,3. Os resultados são discutidos em relação ao provável papel ecológico das ceras e sua aplicação como marcadores taxonômicos.

Palavras chave: ceras epicuticulares; cerrado, floresta semidecídua; alcanos, ecologia.

ABSTRACT- (Foliar epicuticular waxes of congeneric species from forest and cerrado vegetation). Four woody species of cerrado and five woody species of seasonal semideciduous forest were analysed concerning the contents of epicuticular waxes and their parafinic profiles. The cerrado species showed a tendency to higher contents of epicuticular waxes and lower proportion of hydrocarbons than the forest species. The C_{29} and C_{31} alkanes were dominant in all species and the average length of the hydrocarbon chains were around 30,5 in the forest species and from 28,5 to 31,3 in the cerrado species. The ecological and taxonomic aspects of this characteristics are discussed.

Key words: epicuticular waxes; cerrado and semideciduous forest; alkanes, ecology.

Introdução

Todas as plantas terrestres são providas de uma camada hidrofóbica externa à camada cutinizada, de importância vital no sucesso adaptativo dos vegetais na

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conquista do ambiente terrestre, a cera epicuticular. As ceras são misturas complexas diferindo umas das outras em número, abundância relativa e distribuição dos homólogos constituintes de suas classes, sendo a dos álcoois primários uma das mais comuns. Os hidrocarbonetos constituem uma classe de grande ocorrência podendo representar uma alta proporção dos depósitos cerosos (Baker 1982).

Por sua localização entre a planta e o meio, as ceras epicuticulares participam de processos físicos e fisiológicos ecologicamente importantes, tais como controle da perda de água por transpiração (Martin & Juniper 1970) e proteção contra radiação solar, principalmente contra os raios ultravioletas (Kreger 1958). Em um estudo feito com *Cotyledon orbiculata*, Robinson *et al.* (1993) sugeriram que a presença de depósitos cerosos espessos sobre a epiderme conferem uma fotoproteção muito eficiente a plantas expostas a ambientes com alta incidência luminosa. Vários trabalhos ressaltam também a participação das ceras como barreira ao ataque de fungos patogênicos e de herbívoros (Martin & Juniper 1970; Eglinton & Hamilton 1967). Estudos recentes mostram o efeito de repelente alimentar para formigas (saúvas) apresentado por ceras de algumas espécies arbóreas do cerrado (Sugayama & Salatino 1995). Varanda *et al.* (1992) também detectaram efeito deterrente do ácido ursólico da cera de *Jacaranda decurrens* sobre *Schizaphis graminum*. Tal composto provocou também queda na taxa de sobrevivência, no índice de reprodução e na taxa de crescimento populacional dos afídeos.

Muitos autores têm salientado a importância taxonômica do estudo das ceras epicuticulares, principalmente no que diz respeito a sua composição parafínica (Salatino & Salatino 1983, Salatino *et al.* 1989, Blatt *et al.* 1991, Zygodlo *et al.* 1992, Vioque *et al.* 1994).

A vegetação do cerrado foi considerada durante vários anos como xerófila. No final da década de 40 verificou-se que as espécies arbóreas do cerrado transpiravam livremente mesmo durante a estação seca, não sendo a água um fator limitante para as mesmas (Ferri 1944, Rachid 1947, Rawitscher 1948). A partir da década de 50 outros fatores como o oligotrofismo do solo (Arens 1958, Salatino 1993), os altos teores de alumínio (Goodland 1971) e as queimadas periódicas (Coutinho 1976), foram apontados como responsáveis por seu aspecto xeromórfico.

Com relação às ceras epicuticulares, Amaral *et al.* (1985) estudando espécies de dicotiledôneas do cerrado, observaram discreta correlação entre o teor de cera e a espessura da camada de compostos lipofílicos com a pilosidade, porém nenhuma relação entre as características da cera e o grau de escleromorfismo de suas folhas.

Este trabalho tem por objetivo verificar se ocorre alguma variação nos teores de ceras foliares epicuticulares e na composição dos hidrocarbonetos entre espécies relacionadas do cerrado e da mata, a fim de contribuir para o conhecimento do seu papel ecológico e/ou da sua importância como marcador taxonômico.

Material e métodos

Folhas secas ao ar foram submetidas a três extrações sucessivas por imersão de 10 segundos em clorofórmio (Silva-Fernandes 1965). Os extratos foram reunidos,

filtrados e concentrados em rotaevaporador sob pressão reduzida. As ceras obtidas foram pesadas e o rendimento expresso em relação à área foliar ($\text{mg} \cdot \text{dm}^{-2}$).

A composição total da cera bruta foi obtida através de cromatografia em camada delgada ao lado de padrões em placa de sílica gel G60, utilizando clorofórmio como fase móvel. A fração de hidrocarbonetos foi obtida pelo fracionamento da cera bruta por cromatografia em coluna de gel de sílica, sendo eluída com éter de petróleo. Outras misturas de solventes mais polares foram utilizadas na lavagem das colunas para a retirada dos outros componentes da cera.

Os hidrocarbonetos foram identificados por cromatografia gasosa (Cromatógrafo CG-37-D, coluna capilar 10m x 0,25 m, OV 101), por comparação dos tempos de retenção com os de amostras autênticas (Barta & Kómvics 1984). O comprimento médio (CM) das cadeias carbônicas foi calculado de maneira idêntica à determinação de médias em distribuições de frequência.

Resultados e discussão

Os dados obtidos mostram um teor de cera epicuticular muito variável entre as espécies estudadas (Tabela 1). Entretanto, observa-se uma tendência de teores maiores nas espécies coletadas no cerrado comparadas aos seus pares congêneres da mata. Comparando-se as espécies dos gêneros *Styrax* (*S. camporum* - $5,35 \text{ mg} \cdot \text{dm}^{-2}$; *S. sieberi* - $1,41 \text{ mg} \cdot \text{dm}^{-2}$) e *Qualea* (*Q. densiflora* - $6,73 \text{ mg} \cdot \text{dm}^{-2}$; *Q. grandiflora* C - $3,75 \text{ mg} \cdot \text{dm}^{-2}$; *Q. grandiflora* M - $1,44 \text{ mg} \cdot \text{dm}^{-2}$) este teor é cerca de 3,5 vezes maior nas espécies de cerrado. No gênero *Terminalia*, a espécie do cerrado (*T. argentea*) apresentou uma quantidade de cera maior ($2,3 \text{ mg} \cdot \text{dm}^{-2}$) que *T. modesta* ($0,73 \text{ mg} \cdot \text{dm}^{-2}$) e menor que *T. brasiliensis* ($4,05 \text{ mg} \cdot \text{dm}^{-2}$), ambas pertencentes à mata. Em *Rapanea*, a espécie da mata apresentou um teor pouco maior que aquela de cerrado ($1,26 \text{ mg} \cdot \text{dm}^{-2}$ para *R. guianensis* e $1,40 \text{ mg} \cdot \text{dm}^{-2}$ para *R. umbellata*).

Os teores obtidos para as espécies de cerrado analisadas foram menores que aqueles obtidos por Amaral *et al.* (1985) analisando outras espécies de dicotiledôneas deste ambiente. A expressão da quantidade das ceras pode estar relacionada a vários fatores bióticos e abióticos. O teor de cera pode estar muito mais relacionado às características intrínsecas ligadas ao patrimônio genético de cada planta que às condições do ambiente (Bengston *et al.* 1978). Este pode ser o caso das espécies estudadas no presente trabalho.

Uma grande variação também pode ser observada na porcentagem da fração de alcanos nas várias espécies analisadas (Tabela 1). Os maiores valores observados foram encontrados nas espécies de *Qualea*, nas quais esta fração foi a predominante. Outras espécies também apresentaram teores de hidrocarbonetos que podem ser considerados altos, porém não se pode afirmar se esta é a fração predominante nestas espécies por não ter sido feita uma análise quantitativa das demais frações. Não foi observada qualquer relação entre o teor de cera apresentado pelas espécies e a porcentagem de hidrocarbonetos das mesmas. Tulloch (1978) verificou que em *Poa ampla* a presença de alcanos não foi majoritária, havendo uma maior concentração de

hidroxi- β -dicetonas. Lutz & Gülz (1985) verificaram que os hidrocarbonetos representavam a classe de menor expressão nas ceras de sete espécies alpinas.

Tabela 1. Locais de coleta, tipo de vegetação, teor de cera bruta, porcentagem de alcanos e comprimento médio das cadeias carbônicas dos homólogos nas espécies analisadas.

Espécie	Local de coleta	Tipo de vegetação	Teor (mg.dm ⁻²)	% alcanos	CM #
COMBRETACEAE					
<i>Terminalia argentea</i> Mart. & Zucc.	Fz. Sta Carlota *	cerrado	2,30	7,75	30,1
<i>Terminalia brasiliensis</i> Camb.	Fz. Sta Carlota	mata	4,05	16,07	31,1
<i>Terminalia modesta</i> Eichl.	Fz. Sta Carlota	mata	0,73	26,60	25,8
MYRSINACEAE					
<i>Rapanea guianensis</i> Aulb.	Fz. Canchim **	cerrado	1,26	30,65	29,8
<i>Rapanea umbellata</i> (Mart.) Mez.	Fz. Sta Carlota	mata	1,40	29,06	30,1
STYRACACEAE					
<i>Styrax camporum</i> Pohl.	Fz. Canchim	cerrado	5,35	1,62	31,1
<i>Styrax sieberi</i> Perk.	Fz. Sta Carlota	mata	1,41	26,47	30,5
VOCHYSIACEAE					
<i>Qualea densiflora</i> Warm.	Fz. Sta Carlota	cerrado	6,73	71,26	28,8
<i>Qualea grandiflora</i> Mart.	Fz. Sta Carlota	mata	3,75	38,90	30,4
<i>Qualea grandiflora</i> Mart.	Fz. Campininha ***	cerrado	1,44	69,90	30,5

* Município de Cajuru, SP (21° 22'S, 47° 15'W, Alt. 540 a 944m)

** Município de São Carlos, SP (21° 25'S, 47° 51'W, Alt. média 854m)

*** Município de Mogi Guaçu, SP (22° 15' 16"S, 47° 08' 12"W)

Comprimento médio das cadeias carbônicas dos homólogos alcanos

Apesar das porcentagens dos alcanos serem muito variáveis, parece existir uma tendência a valores maiores nas espécies de mata analisadas neste trabalho, excetuando *Q. densiflora* (cerrado) que exibiu o maior valor observado. Esta maior concentração de alcanos nas ceras de espécies de mata (ambiente com alta umidade) pode estar relacionada a características que promovam a repelência de água, visto estas substâncias proporcionarem um maior ângulo de contato (107 - 108°) na interface entre a superfície foliar e a água (Martin & Juniper 1970, Juniper & Jefree 1983). A ausência de água nas superfícies foliares proporciona uma barreira ao desenvolvimento de microorganismos e diminui a resistência à difusão dos gases.

Segundo Harborne & Turner (1984) a razão entre homólogos de número ímpar de átomos de carbono e aqueles de número par frequentemente ocorre na proporção de 10:1. A maioria das espécies analisadas apresentaram uma razão bem maior que esta mencionada acima (Figuras 1 e 2). A presença de constituintes de cadeias mais longas está relacionada a uma maior rigidez do órgão devido ao aumento do ponto de fusão da cera epicuticular, além de contribuir para a eficiência da barreira contra perda de água (Hadley 1981).

Comparando a composição de alcanos das espécies analisadas, aquelas da mata parecem apresentar uma pequena tendência a homólogos de maior comprimento de cadeia quando comparadas aos seus pares congenéricos do cerrado, excetuando o gênero *Styrax*, no qual *S. camporum* apresentou CM maior (31,1) que seu par, *S.*

Figura 1. Composição da fração parafínica das ceras foliares epicuticulares de espécies de *Styrox* e *Qualea*.

sieberi (30,5). Devido às características de escleromorfismo das espécies do cerrado seria esperado que estas apresentassem os maiores valores de comprimento médio das cadeias carbônicas quando comparadas às da mata.

Entre os pares de espécies estudadas foi observada uma grande similaridade quanto ao perfil dos homólogos de hidrocarbonetos presentes, com exceção daquelas do gênero *Terminalia*. Com isso, de maneira geral parece que fatores ambientais não têm grande influência na composição da fração dos hidrocarbonetos nas ceras epicuticulares. As espécies de *Styrox* (Figura 1) e *Rapanea* (Figura 2) apresentaram um predomínio de C29 e C31, além de uma maior expressão do homólogo C33. Em *Qualea grandiflora* o homólogo principal foi C31 enquanto que em *Q. densiflora* foi C29, que representou mais de 50% do total dos hidrocarbonetos (Figura 1).

Dentre os indivíduos analisados, as maiores diferenças interespecíficas foram observadas nas espécies de *Terminalia* (Figura 2). Em *T. argentea* (cerrado) observou-se uma amplitude de composição dos hidrocarbonetos de C27 a C35, com predominância de C29 e C31, enquanto que *T. brasiliensis* apresentou uma distribui-

Figura 2. Composição da fração parafínica das ceras foliares epicuticulares de espécies de *Terminalia* e *Rapanea*.

ção mais restrita com acentuado predomínio de C29 e proporções elevadas de C31 e C33. *T. modesta* apresentou homólogos de cadeia curta (C16 a C19) não detectados nas duas espécies anteriores.

Os dados obtidos concordam com a literatura que afirma que os hidrocarbonetos apresentam predominantemente homólogos com número ímpar de átomos de carbono, entre os quais C29 e C31 são os mais abundantes (Eglinton *et al.* 1962, Eglinton & Hamilton 1967, Douglas & Eglinton 1966).

Com os resultados obtidos conclui-se que dados provenientes das ceras foliares epicuticulares podem ser úteis como subsídio para estudos taxonômicos, principalmente com base na sua fração de alcanos, como já verificado por diversos autores, e que investigações posteriores envolvendo um maior número de pares de espécies dos dois ambientes pesquisados podem fornecer dados interessantes para o entendimento do papel das ceras na natureza.

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Anexo 2

Santos, D.Y.A.C., Pollard, M., Ohlrogge, J. 2006. Labeling of *Arabidopsis* cuticular lipids. **17th International Symposium of Plant Lipids**. p.152.

Labeling of *Arabidopsis* Cuticular Lipids

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The cuticle, being the outermost layer of aerial parts of plants, plays many roles in plant-environment interactions. This layer is composed primarily of waxes and a lipid polyester matrix called cutin. A wide range of mutants is being generated for *Arabidopsis*, and characterized both functionally and by changes in the content and composition of the epicuticular waxes and/or polyester monomers. To better understand the perturbations caused by these mutants will require further pathway and metabolic flux analysis through labeling experiments. Our immediate goal is to assess a variety of labeling protocols in wild-type *Arabidopsis*. A starting point is $^{14}\text{CO}_2$ labeling of intact plants because this represents an unperturbed system compared to other labeling strategies. *Arabidopsis* plants were exposed to 2mCi of $^{14}\text{CO}_2$ for two hours and chased for an additional 70 hours. Leaves and stems were harvested over 3 to 72 hours, epicuticular waxes were extracted by CHCl_3 dip, then soluble (intracellular) lipids extracted, and finally the insoluble residues were transmethyld to release polyester monomers. Labeled lipids were analyzed by TLC and cutin monomers from stems and leaves, namely fatty acids, dicarboxylic acids and ω -hydroxy fatty acids were identified as methyl esters by RP-TLC. The radioactivity in waxes per unit fresh weight was higher in young compared to old leaves, and ~10 fold higher in stems than in leaves. The proportion of radioactivity in waxes and cutin (compared to soluble glycerolipids) increased over the entire chase period indicating that labeling of cuticular lipids is slow compared to labeling of the soluble glycerolipids. This suggests that fatty acid precursors for surface lipids are not supplied directly from plastid export, but rather derive from a pre-existing pool of fatty acids.

Labeling of *Arabidopsis* Cuticular Lipids

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INTRODUCTION

The cuticle is the outermost layer that covers aerial plant surfaces. The major component of this layer in Brassicaceae is a lipid polyester network of hydroxy and dicarboxylic C16 and C18 fatty acids monomers linked by ester bonds (CUTIN). Cutin monomers are synthesized from fatty acyl precursors by oxidases. This cutin matrix contains and is covered by a mixture of long-chain lipophilic compounds (INTRACUTICULAR and EPICUTICULAR WAXES) which are synthesized from C16/C18 saturated fatty acids by elongase complexes. The cuticle layer mediates many interactions between plants and their environment/pathogens/herbivores.

A wide range of mutants is being generated for *Arabidopsis*, and characterized both functionally and by changes in the content and composition of the epicuticular waxes and/or polyester monomers. To better understand the perturbations caused by these mutants will require further pathway and metabolic flux analysis through labeling experiments. Our immediate goal is to assess a variety of labeling protocols in wild-type *Arabidopsis*. A starting point is $^{14}\text{CO}_2$ labeling of intact plants. This is the most natural way to provide label and represents an unperturbed system compared to other labeling strategies.

INITIAL GOALS

1. Can we use radiolabel methods to measure cutin synthesis in intact plants?
2. What is the relationship/timing of wax and cutin accumulation?
3. Can we use radiolabel methods to detect precursor-product relationships of surface lipid synthesis?

MATERIALS & METHODS

RESULTS

1. All wax components were labeled with $^{14}\text{CO}_2$.

The ratio among components is similar with time and similar to the mass of these components on *Arabidopsis* stems. Thus, $^{14}\text{CO}_2$ labeling accurately mimics *in planta* metabolism. Application of ^{14}C -acetate and ^{14}C -palmitate to stem surface also labeled wax components, but ^{14}C -oleate does not (data not shown).

2. Higher amount of ^{14}C -label is accumulated by young than old leaves.

Radioactivity accumulation per unit of fresh weight was always higher in young than old leaves. ^{14}C -Alkane (from wax) accumulation was 6 fold higher in young than in old leaves. ^{14}C -FFA and ^{14}C -DCA (common cutin constituents) were easily detected in young leaves, but not in old. No differences were found in general glycerolipids composition.

CONCLUSIONS

1. $^{14}\text{CO}_2$ labeling of intact *Arabidopsis* plants provides a method to probe surface lipid biosynthesis in undisturbed plants.
2. Young leaves incorporate ~6 fold more label into surface lipid synthesis than old leaves.
3. Incorporation of label into wax occurs in both light and dark for both leaves and stems.
4. Whereas label appeared very rapidly in typical membrane glycerolipids, our data indicate a lag in kinetics of labeling of wax and cutin components. Among cutin monomers, FA is labeled earlier than DCA. Label in DCA component of polyesters occurs only after ~12-24 hr lag.
5. We propose that fatty acid precursors for surface lipids are not supplied directly from plastid export, but likely derive from a pre-existing pool of fatty acids.

3. Wax synthesis continues in the dark.

Plants were labeled for 2 hours in the light and then half were transferred to dark. Leaf fatty acid synthesis essentially stops in the dark (Bao et al., 2000). However, ^{14}C -wax synthesis increased in both light and dark conditions. We suggest this is because a) acyl precursors for wax are derived from a pre-formed pool and b) FA elongation in the cytosol is not light dependent (as previously observed for erucic acid in seeds (Bao et al., 1998)).

4. Acyl Precursors for Surface Lipid Accumulation

After 2 hr pulse with $^{14}\text{CO}_2$, labeling of glycerolipids was rapid and essentially complete by 6 hours (not shown). However, label continued to accumulate in the stem wax fraction for 72 hours. The proportion of radioactivity in waxes (compared to soluble glycerolipids) increased over the entire labeling period indicating that labeling of waxes is slow compared to labeling of the soluble glycerolipids. We interpret these kinetics as indicating that acyl precursors for wax synthesis are not derived directly from plastid export but rather from a pre-existing pool of cellular lipids.

5. Labeling of Cutin Monomers

Cutin monomers were analyzed from the insoluble polyester fraction after transesterification. ^{14}C -labeled FATTY ACIDS appeared after ~12 hr lag. ^{14}C -DCA appeared after ~24 hr lag. These kinetics suggest cutin monomers are not provided directly from plastid export but are derived from a pool of pre-existing fatty acids.

6. Future;

Experiments at longer times will further clarify timing of assembly of cutin monomers. Epidermal lipid composition will be analyzed to identify potential pools of precursors.

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Anexo 3

Santos, D.Y.A.C., Cruz, A., Novaes, L., Almeida, J. 2014. Leaf waxes of Brazilian genotypes of coffee plants (*Coffea arabica* L. – Rubiaceae). **21st International Symposium of Plant Lipids**. p. 46.

Leaf waxes of Brazilian genotypes of coffee plants (*Coffea arabica* L. – Rubiaceae)

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Coffee is an important crop in Brazil. Researches have been discussed about the impact of global changes in this crop productivity. Breeding studies aiming to develop distinct genotypes of coffee plants have been traditionally performed by Agronomic Institute of Campinas (IAC). The role of epicuticular wax profile in improving drought tolerance on economic interest species has been already reported. Our main goal was to investigate the content and composition of leaf waxes of *Coffea arabica* genotypes with different levels of drought tolerance (tolerant – T; susceptible – S or intermediate – I). Leaves from adult field plants were sampled, air dried, and dipped in dichloromethane. The total wax was weighted and an aliquot, added with nonadecane as internal standard, was derivatized with BSTFA and analyzed by GCMS. The foliar area was measured using ImageJ software. The wax layer ranged from 10 to 14 $\mu\text{g}\cdot\text{cm}^{-2}$. However, no correlation between wax thickness and drought tolerance was observed. Caffeine was detected at all samples. Alkanes, fatty acids and triterpenoids were the major compounds (around 60%). Long chain primary alcohols were minority. There is not a clear qualitative distinction among all genotypes. All plants are well established in the field and were submitted to the same watering pattern. We suggest that the absence of differences among the samples is related to plant age and field conditions. Detailed analyzes of cuticular waxes using younger plants, submitted or not to water stress, are in progress. (FAPESP, CAPES, CNPq)

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LEAF WAXES OF BRAZILIAN GENOTYPES OF COFFEE PLANTS *(Coffea arabica L. – RUBIACEAE)*

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INTRODUCTION

Coffee is one of the world's most popular beverage. Only In Brazil, over 5 million people are employed in the cultivation and harvesting of this crop. Two species are mainly cultivated in Brazil - *Coffea arabica* and *Coffea canephora* (Figure 1). Breeding studies aiming to develop distinct genotypes of coffee plants have been traditionally performed. Now a days, one of the biggest challenges is to understand the impact of global changes in this crop productivity. The cuticle is the outermost layer of aerial parts of terrestrial plants, composed by a lipid polyester matrix (cutin) which is surrounded (intracuticular wax) and covered (epicuticular wax) with waxes. Simple chemical constituents in wax are hydrocarbons, aldehydes, wax esters, fatty alcohols, ketones and fatty acids. However, a number of cyclic compounds have also been isolated. The pentacyclic triterpenoids such α - and β -amyrin, ursolic and oleanolic acids are generally the most common. One of the primary functions related to plant cuticle, mainly to wax layer, is the protection against non-stomatic water loss.

GOALS

Our main goal was to investigate the content and composition of leaf waxes of *Coffea arabica* genotypes previously described with different levels of drought tolerance.

MATERIAL AND METHODS

RESULTS AND DISCUSSION

Six genotypes with distinct levels of drought resistance were analyzed. The wax layer ranged from 10 to 17 $\mu\text{g}\cdot\text{cm}^{-2}$. Different then expected, there is no expressive distinction in total wax amount between samples (Figure 2). Despite previous data suggested hydrocarbons, primary alcohols and fatty acids as main compounds (Stocker & Wanner 1975, Kitagami et al. 2013), at the present study all genotypes presented pentacyclic triterpenes as majority (Figure 3).

Figure 2. Total leaf wax ($\mu\text{g}\cdot\text{cm}^{-2}$) of *Coffea arabica* genotypes.

Figure 3. Percentage of main classes of leaf wax compounds. FFA: free fatty acids. HC: hydrocarbons; PA: primary alcohols, PTA: pentacyclic triterpenic acids.

CONCLUSIONS

1. No correlation between wax thickness and drought tolerance was observed;
2. Pentacyclic triterpenoids are the main components of all analyzed coffee genotypes;
3. Looking through total amount and main classes of compounds, there is no difference between leaf waxes of these genotypes;
4. This is the first report of caffeine in coffee's wax.

Figure 1. A: Distribution of two coffee species cultivation; B: Flowering plants of *Coffea arabica*; C: Mature fruits of *Coffea arabica*.

Figure 4. Percentage of main identified compounds in *Coffea arabica* cv. Bourbon Vermelho.

Alkaloids are not common as wax components. Caffeine has been already described in leaf wax of *Ilex paraguariensis*, a South American native species (Athayde et al. 2000). This compound was identified as part of leaf wax of all genotypes. C29 and C31 were the main hydrocarbons, while C30 and C32 were the main primary alcohols (Figure 4). Long chain fatty acids were present as trace (less than 0.5%) in waxes. Among the triterpenes, ursolic acid corresponds to about 80%, followed by oleanolic acid (~13%).

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Anexo 4

Torres, P.B., Chow, F., Santos, D.Y.A.C. 2014. Growth and photosynthetic pigments of *Gracilariopsis tenuifrons* (Rhodophyta, Gracilariaceae) under high light *in vitro* culture. **Journal of Applied Phycology** - DOI 10.1007/s10811-014-0418-z

Growth and photosynthetic pigments of Gracilariopsis tenuifrons (Rhodophyta, Gracilariaceae) under high light in vitro culture

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Growth and photosynthetic pigments of *Gracilariopsis tenuifrons* (Rhodophyta, Gracilariaceae) under high light in vitro culture

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Abstract High levels of irradiance may affect the growth and development of photosynthetic organisms, changing concentrations of carotenoids and chlorophylls. These changes may indicate different photoprotection strategies. In this study, gametophytic apical portions of *Gracilariopsis tenuifrons* were cultivated under controlled laboratory conditions for 1 week, at different light irradiances: 60 (control), 600, and 1,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Growth rate, amount, and composition of pigments were analyzed daily. Color of seaweeds exposed to 600 and 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ varied along the days, from red to yellowish, suggesting a decrease in vital processes as photosynthesis and growth. However, no decrease in biomass was observed. Actually, there was an increase at growth rates for the algae kept under higher light intensities. The main registered pigments were chlorophyll *a*, β -carotene, and zeaxanthin. β -carotene and chlorophyll *a* levels were lower in algae exposed to high light intensity. In treatment exposed to 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, this reduction was 42 and 35 %, respectively, while in those exposed to 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ the values were 55 and 50 % lower than the control. The lower levels of these pigments may be associated with the reduction in energy harvesting by the photosynthetic complexes-antennae, in an effort to dissipate the high excitation impinged over the photosynthesis system as a whole. For zeaxanthin levels, a 20 % increase was observed in the beginning of the experiment, which was followed by a drop to the initial levels, suggesting the role of this pigment in this alga's photoprotection process.

Keywords *Gracilariopsis tenuifrons* · Red algae · Photosynthetic pigments · Growth rate · Light stress · Carotenoids

Introduction

In aquatic environments, high irradiance is common throughout the day, seasons of the year, cycles of sea, and sudden changes in weather (Schubert et al. 2001). In this sense, like any other photosynthetic organism, red seaweeds may be exposed to light incidence rates above the values required by the photochemical processes involved in photosynthesis, leading to light stress, photoinhibition, and photoprotection. In this scenario, the antenna complexes may absorb excess energy, which increase the production of highly unstable molecule known as reactive oxygen species (ROS) (Müller et al. 2001). These unstable molecules may affect the photosynthesis apparatus. In more extreme cases, damage caused by light overexposure may cause cell death or even kill the organism (Osmond 1994). Therefore, high levels of irradiance may considerably affect growth and development of photosynthetic organisms (Taiz and Zeiger 2009; Murchie and Niyogi 2011). These organisms, red seaweeds included, have developed photoprotection mechanisms against light overexposure. Several photoprotection responses regulate photosynthetic light harvesting in order to balance absorption and use of light energy so as to minimize photooxidant damage (Müller et al. 2001). Under intense irradiance, photosynthetic pigments undergo quantitative and compositional changes that are directly associated with distinct photoprotection strategies.

Carotenoids and chlorophyll *a* are present in all organisms that carry out oxygenic photosynthesis, including red algae (Archibald and Keeling 2002; Mimuro and Akimoto 2003). Chlorophyll *a* is present in the photosynthetic reaction center, where it acts in the conversion of solar energy into chemical

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energy, and in antennae, in which the energy is absorbed and transferred to the photosynthetic reaction center. This pigment plays an essential role in ROS formation. High irradiances increase the probability that chlorophyll *a* molecules reach the triplet state (Müller et al. 2001), which in turn may react with free oxygen, forming singlet oxygen, which is one of the main ROS responsible for severe damage to lipids, proteins, and pigments (Krieger-Liszka 2005). On the other hand, under high irradiances, some carotenoids seem to be involved in several photoprotection mechanisms, such as direct ROS deactivation, or as scavengers of triplet chlorophyll, which avoid the formation of singlet oxygen (Dall'Osto et al. 2007). Studies using land plants and green algae have shown that carotenoids are important accessory pigments (Sholes et al. 2011). Nevertheless, in red algae the exact role of these compounds remains to be elucidated.

Since light stress is directly correlated with productivity and nutritional quality of commercial cultivations, light responses of photosynthetic organisms have considerable economic and scientific interest. Additionally, light responses represent a source of essential information to better understand the biology and distribution of natural populations, broadening the panorama of the effects of climate change at global level, and the ecophysiology of species, favoring the sustainable, rational management and use of natural resources (Hanelt 1996; Taiz and Zeiger 2009).

In this scenario, the present study evaluates how the increase of photosynthetically active radiation (PAR) affects photosynthetic pigments (carotenoids and chlorophyll *a*) and growth rates of the red seaweed *Gracilariopsis tenuifrons* (C.J. and E.C Oliveira) Fredericq and Hommersand under laboratory conditions. The results obtained should help better understand the biology of this species and to evaluate the behavior of these pigments under light stress.

Materials and methods

Materials and general growth conditions

Apical tips of *Gracilariopsis tenuifrons* female gametophytic phase (haploid) (BG0039, Germplasm Bank) were acclimatized for 1 month under controlled conditions (25 ± 1 °C; photosynthetic photon flux density (PPFD) of 60 ± 5 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; 14-h light/10-h dark photocycle, intermittent aeration at 30-min intervals) using sterilized seawater (32 psu) enriched with von Stosch solution 50 % (Ursi and Plastino 2001 based on Edwards 1970). The light was provided by fluorescent lamps (model day light, 40 W), and the irradiance was determined using a PPFD spherical sensor (LI-COR Model LI-1935B) connected to a recorder (LI-COR Model LI-250). The unialgal culture was done in the Laboratory of

Marine Algae Edison José de Paula, Institute of Biosciences, University of São Paulo.

Experimental design and growth

Acclimatized apical portions of 3 cm in length were submitted to three treatments of PPFD: 60 μmol (control), 600 μmol , and 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in Erlenmeyer flasks. All other cultivation conditions were the same described above. The algae biomass and seawater inside each Erlenmeyer flask was 1 g L⁻¹. Seven sets of three (replications) Erlenmeyer flasks, with known initial mass (M_i), were prepared for each PPFD treatment. Daily, during the 1-week experimental period, three flasks (replication) from each light treatment were analyzed. These flasks were sampled, and final algal biomass (M_f) was recorded to calculate the growth rate (GR) as $\text{GR (\% day}^{-1}\text{)} = [(M_f/M_i)^{1/t} - 1] \times 100$ in which M_f =final biomass (g), M_i =initial biomass (g), t =day of sampling (Lignell and Pedersen 1989; Yong et al. 2013). After weight, samples of 50 mg (fresh weight-FW) of apical portions of each flask were retrieved, frozen in liquid nitrogen and stored at -80 °C for subsequent pigment analyses.

Extraction, analyses, and quantification of carotenoids and chlorophyll *a* by HPLC

Extraction of carotenoids and chlorophyll *a* was carried out using the frozen samples of 50 mg FW described above. The material was triturated in liquid nitrogen. Next, 1.5 mL methanol was added, the mixture was homogenized and centrifuged (28,800×g, 4 °C, 5 min) (Torres et al. 2014). Aliquots of the supernatant were then immediately analyzed by high performance liquid chromatography (HPLC) in a chromatograph (HP1200) equipped with a reverse-phase C₃₀ column (Ultrasorb ODS, 250×4.6 mm i.d., 5 μm). The chromatograms obtained were processed at $\lambda=450$ nm. The mobile phase was a gradient of methyl-terc-butyl ether (MTBE) in methanol following the program: 5 % MTBE (0 min), 70 % MTBE (30 min), and 50 % MTBE (50 min). The mobile phase flux was kept constant at 0.9 mL min⁻¹ and the column temperature was adjusted to 29 °C (Faria et al. 2009).

The pigments were quantified by HPLC using external calibration curves constructed for chlorophyll *a* and zeaxanthin. The curves were constructed using standard solutions of known concentrations and analyzed under the same conditions as the samples.

Characterization of the photosynthetic pigments

Carotenoids and chlorophyll *a* were characterized using HPLC coupled to a mass spectrometer (HPLC-MS/MS) with an ion trap analyzer and atmospheric pressure chemical ionization (APCI) ionization source (Shimadzu, LC-20 AD; Bruker Daltonics, Esquire 4000, Germany). The UV/visible spectra were obtained

at between 200 and 800 nm, and chromatograms were processed at λ 450 nm. The parameters of the mass spectrometer were adjusted following Rosso and Mercadante (2007): APCI positive mode, corona current 4,000 nA, source temperature 450 °C, N_2 as dissecting gas at 350 °C and 5 mL min⁻¹ and as nebulizing gas at 414 kPa, and MS/MS fragmentation energy 1.4 V. Mass spectra were acquired at a 100–1000 m/z interval. Separation of carotenoids was carried out in a C₃₀ YMC column (250×4.6 mm i.d., 5 μ m) (Waters, USA) using the same mobile phase as described above.

Carotenoids and chlorophylls were identified in HPLC-MS/MS according to retention times in the C₃₀ column, based on the UV/visible (λ_{max} , fine structure, *cis* peak structure) and mass spectra, comparison with literature data (Gauthier-Jaques et al. 2001; Breemen et al. 2011), and comparison of retention times with those of standards (chlorophyll *a* and β -carotene were purchased from Sigma-Aldrich; other carotenoids were supplied by CaroteNature).

Statistical analyses

The one-factor analysis of variance (ANOVA) was used to determine the significant differences between means of each variable on a daily basis, between treatments. Existing differences were analyzed using the post-hoc Tukey test. Additionally, variations in pigment concentrations in apices exposed to the light regimens with time were analyzed by single linkage hierarchical clustering with Pearson correlation (1-r) distance index. All analyses were carried out considering $\alpha=5\%$ using Minitab 16.1.0, except the multivariate analyses, which was conducted using Statistica 10. The treatments were carried out in triplicate.

Results

Effects of irradiance on the general aspect of growth

In the control group (60 μ mol photons m⁻² s⁻¹), apices did not present variation in color throughout the experiment. Control apices analyzed on day 7 presented similar color to that of apices analyzed on day 0. However, exposure to the other two experimental light levels (600 and 1000 μ mol photons m⁻² s⁻¹) led to phenotypic changes manifested as variation in color, from reddish to yellowish. This variation was more intense in the apices exposed to the highest irradiance, and seemed to build up throughout the experimental period. Apices exposed to 1000 μ mol photons m⁻² s⁻¹ for 7 days were the most affected, becoming totally yellow (Fig. 1).

As a rule, the increase in irradiance raised growth rates of exposed apices from day 1. The growth rates were of

11.7 % day⁻¹ for apices exposed to 600 μ mol photons m⁻² s⁻¹ (Fig. 2b) and 12.6 % day⁻¹ for apices exposed to 1000 μ mol photons.m⁻² s⁻¹ (Fig. 2c), in comparison with control (6.4 %.day⁻¹) (Fig. 2a). For the control group, a significant increase between growth rates measured on day 1 (6.4±0.08 % day⁻¹) and those measured on day 3 (9.8±1.37 % day⁻¹) was observed, which remained constant throughout the rest of the experiment. Similar results were observed for apices exposed to 1000 photons m⁻² s⁻¹ at the beginning of the experiment, whose growth rates increased between day 1 (12.6±1.75 % day⁻¹) and day 3 (15.9±0.91 % day⁻¹). However, from day 4 on (13.7±0.84 % day⁻¹), a significant decrease in growth rates was recorded until day 6 (12.7±1.16 % day⁻¹) and day 7 (13.5±0.65 % day⁻¹), when they became identical to the measurements carried out on day 1. Growth rates of apices exposed to 600 μ mol photons m⁻² s⁻¹ did not vary significantly, remaining essentially constant during the experiment. These results indicate that apices exposed to the three irradiance values grew along the experimental period. However, even though the apices exposed to 1000 μ mol photons m⁻² s⁻¹ experienced a slowing down of growth rate at the end of the experiment, these apices did not stop growing. They only grew at a slower pace.

Effects of irradiance on pigment composition

β -carotene (all-*trans*- β -carotene), β -cryptoxanthin (all-*trans*- β -cryptoxanthin), zeaxanthin (all-*trans*-zeaxanthin), and chlorophyll *a* were identified in the extracts of the macroalga studied (Fig. 3). The main pigments present in the extract were chlorophyll *a* and zeaxanthin, which was the main carotenoid detected in apices exposed to all experimental light irradiance values. The carotenoid β -cryptoxanthin was highly variable among replicates; when present, it was at low concentrations (under 1 % of the total area). No changes in pigment composition were observed across the apices exposed to the three light levels. Yet, quantitative changes in response to higher irradiance values were significant.

No significant changes were observed for photosynthetic pigments in apical portions cultivated as control group. β -carotene, zeaxanthin, and chlorophyll *a* remained constant throughout the seven day-experiment (means 52.5±3.1 μ g g⁻¹, Fig. 4a; 77.1±3.8 μ g g⁻¹, Fig. 4b; and 495.4±27.8 μ g g⁻¹, Fig. 4c, respectively). Concentration of zeaxanthin was 1.5 times as high as that of β -carotene, while levels of chlorophyll *a* were roughly 3.8 times higher than total carotenoid concentrations (zeaxanthin+ β -carotene).

On the other hand, higher irradiances led to a large reduction in chlorophyll *a* and β -carotene levels. Chlorophyll *a* levels in apices exposed to 600 μ mol photons m⁻² s⁻¹ remained constant until day 3. From then on, significant

Fig. 1 Apices of *Gracilariopsis tenuifrons* after exposure to 60, 600, and 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ throughout the growth period. The numbers above columns indicate the time, in days, of the experiment (day 0–7)

decreases were recorded, until day 7, when chlorophyll *a* levels in these apices were 35 % lower, compared to the beginning of the experiment (day 0) (Fig. 4f). The same was observed for apices exposed to 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, with a significant linear reduction ($R^2=0.998$, simple linear regression) during the experiment, accounting for over 50 % on day 7, compared to day 0 (Fig. 4i). Regarding β -carotene levels in apices exposed to 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, significant decreases were observed as early as on day 2. The largest drop, of around 42.9 %, was recorded on day 7, compared to day 0 (Fig. 4d). As for apices exposed to 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, a significant linear decrease ($R^2=0.989$, simple linear regression) was noticed during the experiment, which summed up to more than 55 % (Fig. 4g). Therefore, increased irradiances decrease β -carotene levels in a steep fashion.

Zeaxanthin levels in apices exposed to 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ showed a large increase, of around 20 %, between day 0 ($68.9 \pm 2.1 \mu\text{g g}^{-1}$) and day 2 ($82.1 \pm 3.7 \mu\text{g g}^{-1}$). From day 3 on, zeaxanthin levels began to drop, reaching a value similar to day 0 on day 7 (Fig. 4e). At the beginning of the 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ regime, zeaxanthin levels presented the same pattern observed in apices exposed to 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, though they increased significantly (by 20 %) between day 0 ($76.6 \pm 2.0 \mu\text{g g}^{-1}$) and day 3 ($92.5 \pm 3.1 \mu\text{g g}^{-1}$). However, the reduction in concentration of this carotenoid was more evident in apices exposed to the highest irradiance (1,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), and fell from day 4 on, reaching the lowest value on day 7, (7.9 % lower than on day 0; Fig. 4h).

In spite of the significant decrease in zeaxanthin levels after exposure to high irradiances, the zeaxanthin/ β -carotene and

Fig. 2 In vitro growth rates ($\% \text{ day}^{-1}$) (means $\pm$ SD) of apices of *Gracilariopsis tenuifrons*, on a daily basis, exposed to different irradiances (60, 600, and 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Identical letters on one same curve indicate that values did not differ in the one-factor ANOVA and in the Tukey test, ($p < 0.05$)

Fig. 3 HPLC chromatogram of methanol extract of *Gracilariopsis tenuifrons*. The numbers above each peak represent Zeaxanthin (1), β-Cryptoxanthin (2), Chlorophyll *a* (3), and β-carotene (4)

zeaxanthin/chlorophyll *a* ratios increased throughout the experimental period (Table 1). Concerning the zeaxanthin/β-carotene ratio, exposure to 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ led to a 74 % increase as measured on day 7, compared to day 0, while 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ caused an

increase of 114 % for the same period. As for the zeaxanthin/chlorophyll *a* ratio, exposure to 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ caused a 57 % increase, while exposure to 1000 increased this by 87 %. A trend towards decrease was observed in the β-carotene/chlorophyll *a* ratio in apices exposed to the highest irradiance (Table 1). When compared to day 0, a 9 % decrease was observed in this ratio for apices exposed to 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, while for apices exposed to 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ this decrease was 8 %, on day 7. These results indicate that zeaxanthin levels decrease at a lower rate, when compared to β-carotene and chlorophyll *a* over the experimental period.

For the control group, zeaxanthin/β-carotene, zeaxanthin/chlorophyll *a*, and β-carotene/chlorophyll *a* ratios remained essentially constant throughout the experimental period (means 1.40 ± 0.03 , 0.15 ± 0.00 , and 0.11 ± 0.00 , respectively).

Analyses of hierarchical clustering

In order to facilitate the global comprehension of the behavior of pigments with exposure to different irradiances, a hierarchical cluster analysis was carried out (Fig. 5). The parameters

Fig. 4 Effect of the different irradiances (60, 600, and 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) on β-carotene, zeaxanthin, and chlorophyll *a* concentrations ($\mu\text{g g}^{-1}$) (means $\pm$ SD, $n=3$) in apices of *Gracilariopsis tenuifrons*. Identical letters on one same curve indicate that values did not differ in the one-factor ANOVA and in the Tukey test ($p < 0.05$)

Table 1 Effect of the different irradiances (600 and 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) on the zeaxanthin/ β -carotene (z/ β), zeaxanthin/chlorophyll *a* (z/Chl), and β -carotene/chlorophyll *a* (β /Chl) ratios (means $\pm$ SD) in apices of *Gracilariopsis tenuifrons* over the experimental period

Time (day)	600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$			1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$		
	z/ β	z/Chl	β /Chl	z/ β	z/Chl	β /Chl
0	1.53 $\pm$ 0.04 ^a	0.16 $\pm$ 0.00 ^a	0.11 $\pm$ 0.00 ^a	1.56 $\pm$ 0.01 ^a	0.16 $\pm$ 0.00 ^a	0.10 $\pm$ 0.00 ^a
1	1.58 $\pm$ 0.01 ^a	0.18 $\pm$ 0.00 ^{ab}	0.11 $\pm$ 0.00 ^a	1.79 $\pm$ 0.07 ^a	0.20 $\pm$ 0.00 ^b	0.11 $\pm$ 0.00 ^d
2	1.84 $\pm$ 0.03 ^{ab}	0.20 $\pm$ 0.00 ^{bc}	0.11 $\pm$ 0.00 ^a	2.30 $\pm$ 0.04 ^b	0.24 $\pm$ 0.00 ^c	0.10 $\pm$ 0.00 ^a
3	2.06 $\pm$ 0.13 ^{bc}	0.22 $\pm$ 0.01 ^{cd}	0.11 $\pm$ 0.00 ^a	2.50 $\pm$ 0.08 ^{bc}	0.25 $\pm$ 0.00 ^{cd}	0.10 $\pm$ 0.00 ^a
4	2.12 $\pm$ 0.23 ^{bc}	0.21 $\pm$ 0.01 ^{cd}	0.10 $\pm$ 0.01 ^b	2.66 $\pm$ 0.06 ^c	0.26 $\pm$ 0.00 ^{cde}	0.10 $\pm$ 0.00 ^{ab}
5	2.30 $\pm$ 0.16 ^{cd}	0.22 $\pm$ 0.01 ^d	0.10 $\pm$ 0.01 ^b	2.77 $\pm$ 0.01 ^{cd}	0.28 $\pm$ 0.00 ^{def}	0.10 $\pm$ 0.00 ^{ab}
6	2.33 $\pm$ 0.16 ^{cd}	0.23 $\pm$ 0.01 ^d	0.10 $\pm$ 0.00 ^b	3.06 $\pm$ 0.09 ^{de}	0.29 $\pm$ 0.00 ^{ef}	0.09 $\pm$ 0.00 ^c
7	2.68 $\pm$ 0.13 ^d	0.26 $\pm$ 0.01 ^e	0.10 $\pm$ 0.00 ^b	3.20 $\pm$ 0.27 ^e	0.31 $\pm$ 0.02 ^f	0.10 $\pm$ 0.00 ^{bc}

Identical letters on one same column indicate that values did not differ in the one-factor ANOVA and in the Tukey test, ($p < 0.05$)

included daily means of each pigment's concentrations after treatments.

Algae grown in the control group (60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) (group 1) separated from those exposed to light stress (600 μmol and 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) (group 2) (Fig. 4). In the cluster formed by algae exposed to light stress, subclusters formed with high correlation ($r \geq 0.95$) between chlorophyll *a* and β -carotene, independently of the treatment (group 4), which indicates that these pigments exhibited similar behavior during the experiment.

In turn, the response pattern exhibited by zeaxanthin was quite different from those observed for chlorophyll *a* and β -carotene at each treatment of light stress. However, the similar responses observed for zeaxanthin in both high irradiance

treatments, that is, an initial increase followed by a drop in concentration, led to the formation of group 3.

Discussion

The growth rate of control treatment from this study was higher than that observed in experimental field cultures of *G. tenuifrons* in Venezuela ($3.63 \pm 0.50 \% \text{ day}^{-1}$; Gómez and Millán 1997) and in Colombia ($0.59 \pm 0.39 \% \text{ day}^{-1}$; Rincones and Moreno 2011). Additionally, the growth rate was almost twice than the growth rates observed in laboratory conditions for *Gracilariopsis lemaneiformis* ($4 \pm 0, 5 \% \text{ day}^{-1}$; Zou and Gao 2014) and *Gracilariopsis longissima* ($3.03 \pm 0.11 \% \text{ day}^{-1}$; Qing et al. 2014), indicating a superior physiological performance than reported for the same and similar species.

Zeaxanthin was the main carotenoid in apices of *Gracilariopsis tenuifrons* exposed to control treatment and to high irradiance treatments. This observation is distinct from what has been reported for leaves of land plants and for most red algae, which have lutein as the main carotenoid (Young 1993; Marquardt and Hanelt 2004; Schubert et al. 2006). Other studies with different species of *Gracilariopsis* also detected zeaxanthin as the main carotenoid. However, distinctly from the results observed here, violaxanthin and anteraxanthin were also detected in *G. lemaneiformis* (Schubert et al. 2006) and, for two other species, components of α -carotene biosynthetic pathway were also present (Andersson et al. 2006). Thus, based on our results and previous data for different red algal species, there is a considerable difference between carotenoid profiles in these organisms. These findings contrast with what has been reported for leaves of land plants, in which carotenoid compositions are consistently similar between the different species analyzed, with lutein being predominant (Young 1993; Britton 2008).

Fig. 5 Hierarchical clustering dendrogram constructed using the different pigment compositions over the experimental period (day 0–7) for the different irradiances used. Z zeaxanthin, β β -carotene, c chlorophyll *a*. The numbers associated with each pigment refer to the irradiances 60, 600, and 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, while the numbers 1–4 describe the clusters formed. Distances were estimated as 1-Pearson *r*

It is known that carotenoids exhibit different photoprotection responses (Britton 2008). In this sense, this diversity in pigment composition observed for red algae may indicate behavioral differences in response to high irradiances across different species with distinctive pigment composition.

Under the conditions of light exposure studied here, variations in pigment concentrations in *G. tenuifrons* were observed, but compositional differences were not detected. The concentrations of the pigments chlorophyll *a* and β -carotene were reduced, and were inversely correlated with irradiance. Similar results have been obtained for other red algae species, such as *Gracilaria tenuistipitata* var. *liui* (Carnicas et al. 1999) and *Chondrus crispus* (Yakovleva and Titlyanov 2001).

The responses of these pigments were highly correlated ($r \geq 0.95$), suggesting that these compounds were affected, in a similar way, by the increase in irradiance. Reductions in the area of thylakoids, in the number of photosystems or in the size of antennae may be considered as different photoprotection strategies that affect both chlorophyll *a* and β -carotene in a similar way. These strategies have been described for the red microalga *Porphyridium cruentum* (Cunningham et al. 1989).

In land plants and green algae, it is well known that chlorophyll *a* and β -carotene are involved in harvesting light in antennae (Ritz et al. 2000). Therefore, in a scenario of high light intensity, it is possible to suppose that a decrease in these pigments reduces energy absorption in the antennae, lessening the excitement effect on the photosynthetic system as a whole. The real role of chlorophyll *a*, and more precisely, of β -carotene in photosynthetic process for red seaweeds is not totally known, even less so at stress conditions. However, since we observed a marked decrease of these pigments under high light intensity culture, it is not impossible to suggest that they could be involved in harvesting light in antennae. The decrease observed could be a way to avoid the over excitement of the photosynthetic system.

The behavior of the carotenoid zeaxanthin differed from that exhibited by chlorophyll *a* and β -carotene. Under high irradiance, zeaxanthin concentrations rose in the beginning of the experiment, followed by a slight decrease towards the end. In spite of this falling trend, the zeaxanthin/chlorophyll *a* and zeaxanthin/ β -carotene ratios increased consistently throughout the experiment, indicating that zeaxanthin production was higher, as compared to other pigments, during acclimation. Under this perspective, it is possible to suppose that zeaxanthin plays an important protective role in this algal species. These results can be corroborated by analyzing the ratios of zeaxanthin and β -carotene or chlorophyll *a* under 600 and 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, in which a significant increase of z/β and z/Chl ratios indicate the increase of zeaxanthin concentration probably due to the photoprotection function of this antenna pigment as a thermal dissipator of excess absorbed light energy under high irradiance, a process

identified as the xanthophyll cycle, a process which is little known and still uncertain in red macroalgae.

In the literature, several studies have assessed the photoprotective role of zeaxanthin, indicating that this pigment is efficient in eliminating chlorophyll triplet, oxygen singlet, and other ROS (Havaux and Niyogi 1999; Betterle et al. 2010; Dall'Osto et al. 2010). Several studies also suggested that the antioxidant capacity of zeaxanthin is higher than that of other xanthophylls (Havaux et al. 2007). Schubert and Garcia-Mendoza (2008) studied different red algae with distinct carotenoid compositions and concluded that the species richer in zeaxanthin exhibit less light sensitivity. Other studies on red algae, such as *G. tenuistipitata* (Carnicas et al. 1999) and *Corallina elongata* (Esteban et al. 2009) also have reported an increase in zeaxanthin levels at higher irradiances in long-term experiments. Additionally, in land plants and green algae, experiments using mutants that accumulate zeaxanthin have demonstrated that this pigment is an efficient antioxidant, and that it plays a role in the protection of lipo-protein membranes against peroxidation (Havaux et al. 2007).

In land plants and green algae, the photoprotective role of zeaxanthin is associated mainly with its presence in the outer antennae of PSII and, more specifically, with the xanthophyll cycle, as shown by Niyogi et al. (1998), Holt et al. (2004), and Dall'Osto et al. (2005), among others. Nevertheless, it is known that the outer antennae of red algae are quite different, since these are formed by a protein-pigment complex extrinsic to thylakoid membranes, which are known as phycobilisomes, and which do not contain zeaxanthin (Grossman et al. 1993). This difference supports the uncertainty surrounding the functional presence of a typical xanthophyll cycle in red algae, or the role of this pigment in immediate thermal dissipation, for instance.

Phycobiliproteins, the characteristic reddish pigment of red macroalgae, are the most sensitive antenna pigment, localized externally to the reaction center and the first photosynthetic pigment to decrease in concentration under light stress (Bouzon et al. 2012; Gouveia et al. 2013; Santos et al. 2014). Under the most extreme condition of this experiment (day 7, 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), the apical portions of *G. tenuifrons* were completely yellowish (Fig. 1). Decreases in pigment concentration associated with dramatic loss of color under high irradiances have been described, mainly for land plants, as a sign of chronic photoinhibition (Powles 1984; Osmond 1994). At this stage, the photosynthesizing capacity and consequently the biomass are reduced, indicating the occurrence of severe damage to metabolism. In the present study, however, the growth rates observed show that this alga continues to grow, even when exposed to higher light intensities. Therefore, it is possible to suggest that the loss of pigmentation by *G. tenuifrons*, at least in the experimental design adopted here, is not necessarily associated with severe damage to the photosynthetic apparatus; on the contrary, the

loss of color may reflect photoacclimation and photoprotection strategies.

Quintano et al. (2013), in a field study with *Gelidium corneum*, suggested that pigment lost could be associated with nitrogen deficiency, resulting in more fragile individuals. In our study, nitrogen supply was tentatively kept the same during the week and, at least for the treatment of 600 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, more rigid individuals were observed. Morphological similarities between our results and Quintano et al. (2013) were verified only by the end of the 1000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ culture treatment, associated with a reduction on the growth rate. It suggests that, above a critical point, light intensity turns prejudicial to algae development.

The loss of the reddish color in red algae may be common in scenarios of high irradiance. In the field, for instance, red algae may appear yellowish or brownish in summer, as opposed to the deep red color observed in winter (Jones and Williams 1966; Waaland et al. 1974). The increased number of soft-pigmented algae in the field has been pointed out as consequence of climatic changes (Díez et al. 2012). Thus, understanding the mechanisms of photoprotection of these organisms is an important subject from an ecological and economical point of view.

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Anexo 5

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Influence of Viral Infection on Essential Oil Composition of *Ocimum basilicum* (Lamiaceae)

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Ocimum basilicum L., popularly known as sweet basil, is a Lamiaceae species whose essential oil is mainly composed of monoterpenes, sesquiterpenes and phenylpropanoids. The contents of these compounds can be affected by abiotic and biotic factors such as infections caused by viruses. The main goal of this research was an investigation of the effects of viral infection on the essential oil profile of common basil. Seeds of *O. basilicum* L. cv. Genovese were sowed and kept in a greenhouse. Plants presenting two pairs of leaves above the cotyledons were inoculated with an unidentified virus isolated from a field plant showing chlorotic yellow spots and foliar deformation. Essential oils of healthy and infected plants were extracted by hydrodistillation and analyzed by GCMS. Changes in essential oil composition due to viral infection were observed. Methyleugenol and *p*-cresol, 2,6-di-*tert*-butyl were the main constituents. However, methyleugenol contents were significantly decreased in infected plants.

Keywords: essential oil, *Ocimum basilicum*, sweet basil, plant virus, methyleugenol.

The genus *Ocimum* L. (Lamiaceae) comprises 30 - 160 annual and perennial herbs and shrubs, collectively called basil. Species of this genus are popular sources of essential oils and aromatic compounds, of condiments, and ornamental plants. The most cultivated species worldwide are *O. africanum* Lour., *O. americanum* L., *O. basilicum* L., *O. gratissimum* L., *O. minimum* L. and *O. tenuiflorum* L., mainly due to their economic and medical importance [1].

Ocimum basilicum L., popularly known as common or sweet basil, is an annual crop widespread in Asia, Africa, South America and the Mediterranean region. It is widely cultivated in many countries under natural and greenhouse conditions [2]. *O. basilicum* leaves are used in infusions and decoctions for sore throats, dizziness, convulsion, colds and vomiting [3]. Sweet basil is also used as a raw material for the essential oil and drug industries [4,5]. In Brazil, this species is used as a food condiment either *in natura* or manufactured, and for essential oil extraction [6].

O. basilicum essential oil is constituted of phenylpropanoids, like eugenol, chavicol and its derivatives, and terpenoids, like limonene, linalool and methyl cinnamate [7]. Masi *et al.* [8], studying nine

different cultivars of *O. basilicum* commonly utilized in the Mediterranean area, were able to identify five distinct chemotypes based on the main essential oil constituent. Iso-pinocamphone (35.1%) and carvone (39.7%) were the predominant components of the essential oil in cultivated *O. basilicum* at the Garden of Medicinal Plants in Salerno [9].

Essential oils are influenced by environmental (drought, temperature) and biological factors (pathogens, herbivores). These might be responsible for a decrease in the crude essential oil amount, and for changes in their composition [10].

Virus infecting plants are pathogens responsible for considerable economic losses [11]. A comparative analysis of essential oil yields from fresh aerial parts of *Lavandula hybrida* "Alardi" (Lamiaceae) showed that AMV (Alfalfa mosaic virus) infection reduced the volatile oil fraction by 36%, with a net decrease in monoterpene content accompanied by a 50% increase in sesquiterpenes [12].

The natural occurrence of Cucumber mosaic virus (CMV) and Broad bean wilt virus (BBWV) associated with chlorotic spots and leaf distortion in basil has already been

reported [13]. Ring spots, leaf distortion, and severe mosaic symptoms induced by Tomato spotted wilt virus [14], and interveinal chlorosis caused by Pepino mosaic virus (PepMV) on basil (*O. basilicum*) were also reported [15]. Despite these reports, no data were available about essential oil composition resulting from viral infection of sweet basil. Thus, the main goal of this research was to investigate the influence of natural virus infection on the composition of *O. basilicum* essential oil.

Mechanical transmission of isolated field-basil virus did not induce visible symptoms on healthy basil plants in laboratory experiments. Failure in mechanical transmission of the virus can be related to host plant conditions, environmental factors, and/or virus concentration in the inoculum [17]. The presence of inhibitory substances can also be addressed as a possible reason. The virus was not transmitted after aphid-transmission assay in a non-persistent manner. Notwithstanding, healthy basil plants submitted to aphid-transmission assay in a circulative manner developed the same symptoms observed on naturally infected plants, detected as chlorotic yellow spots and leaf distortion.

Among the aphid-transmitted viruses in a circulative manner are species of *Luteoviridae* (*Luteovirus*, *Polerovirus* and *Enamovirus*), *Nanoviridae* (*Nanovirus* and *Babuvirus*), *Rhabdoviridae* (*Cytorhabdovirus* and *Nucleorhabdovirus*) and *Umbravirus* [18,19]. The natural occurrence of the above mentioned viruses on *O. basilicum* has not been reported so far. However, based on some properties of the virus under study in the present research, for example, the failure of mechanical transmission, the successful transmission by *M. persicae* in a circulative manner, and the induction of foliar yellow chlorosis, the presence of a *Luteoviridae* virus can be suggested [19].

Many members of the *Luteoviridae* are phloem-limited, causing 'yellow'-type diseases [19]. Moreover, Tomas and Hassan [20] reported *O. basilicum* as an experimental host for Potato leafroll virus (*Polerovirus*), a member of the *Luteoviridae* family.

Previous studies have already reported rigid particles associated with chlorotic spots for *Ocimum* sp in Brazil [16]. However, in the present study, transmission electron microscopy was not able to unveil any virus particle from naturally infected *O. basilicum*, probably due to a low virus concentration in the plant.

Based on available data, the virus from the studied basil remains unidentified, although a member of the *Luteoviridae* might be involved. Further analyses using common host plants susceptible to aphid transmission, different buffer solutions in the mechanical transmission assay, and serological and molecular tests [19] are being conducted.

Although the virus has not been identified, it is well known that viral infection can induce varied plant metabolism changes, modifying respiration rates, photosynthesis, and transpiration [17]. However, there are few papers concerning secondary metabolism changes in virus infected plants [12, 21-23].

Both, healthy and infected plants of *O. basilicum* cv. Genovese presented methyleugenol and *p*-cresol,2,6-di-*tert*-butyl as main constituents (Table 1). Essential oil analyses of *O. basilicum* cv. Genovese Gigante from different regions in Italy showed methyleugenol and eugenol as main constituents. Besides these compounds, linalool, cineole, camphene and cadinene were also found [24]. In the present study, linalool, eugenol, *trans*- α -bergamotene, bergamotene, 3-carene, β -ocimene, β -farnesene, δ -cadinene and (Z,E)- α -farnesene were also detected (Table 1).

Quantitative comparison concerning the two main constituents showed a significantly larger amount of methyleugenol in healthy plants than in infected ones. Even though an increase in *p*-cresol,2,6-di-*tert*-butyl amount has been observed in infected plants, no significant difference was detected (Figure 1). Essential oil yield reduction and qualitative differences were also observed in *Salvia sclarea* infected by Broad bean wilt virus (BBWV) [21].

Table 1: Composition of essential oil of *Ocimum basilicum* L. cv. Genovese analyzed by GC-MS.

Constituents	RT (min)	Healthy plants					Infected plants				
		1	2	3	4	5	1	2	3	4	5
Linalool	6.44	9.8									
Eugenol	11.84	5.9	2.1	7.1	4.9	2.1					
Methyleugenol	12.92	35.6	43.2	31.7	30.6	48.5	14.0	14.0	13.1	31.0	24.1
<i>Trans</i> - α -bergamotene	13.50		4.4	1.9	6.1	4.5					
Bergamotene	13.53						13.1	6.1		13.0	
3-Carene	13.73		1.8		1.5						
β -Ocimene	13.77						4.1				
β -Farnesene	14.03		1.9		2.4		5.6				
<i>p</i> -Cresol,2,6-di- <i>tert</i> -butyl	15.03	46.9	43.1	55.3	49.5	44.9	57.5	77.6	66.2	49.1	64.5
δ -Cadinene	17.40	1.7			1.7						
(Z,E)- α -Farnesene	21.55		3.5								
N.I.*				3.9	3.3		5.7	2.3	20.7	6.9	11.4

* N.I. – Not identified (corresponds to the total non identified constituents from the sample).

Figure 1: Mean and standard deviation of the main compounds detected in healthy and infected plants of *Ocimum basilicum* cv. Genovese. Different letters mean significant differences ($p \leq 0.05$).

Qualitative differences were observed between healthy and infected plants. Some constituents, such as linalool and eugenol, were found only in healthy plants, while others, like bergamotene, were detected only in infected plants (Table 1). Variability in essential oil amount and composition related to pathogen infection was also detected in *Hypericum perforatum* L. infected by phytoplasma [10]. Reduction in essential oils after viral infection has been pointed out for Lamiaceae species, for example, *Salvia sclarea*, *Lavandula vera* and its hybrids, and *Agastache anethiodora* [12,21,22]. Duarte *et al.* [23] observed a decrease in total phenolics and alkaloids from virus-infected *Datura stramonium*.

Higher amounts of non-identified compounds were detected in infected plants than in healthy ones. Plant defense mechanisms, which include new genes activation, might be correlated to the production of new compounds and/or their accumulation. This defense response is a determinant event in plant protection [25,26]. These results reveal that sweet basil plants infected by natural-basil virus transmitted by aphids in a circulative manner have changed the essential oil composition, with a significant decrease in methyleugenol production, an apparent increase in *p*-cresol, 2,6-di-*tert*-butyl, and higher amounts of non-identified compounds.

Experimental

Plant material: Seeds of sweet basil (*Ocimum basilicum* L. cv. Genovese) were sowed on a tray filled with

sterilized soil, kept inside a greenhouse, and watered twice a day. Seedlings with 2 pairs of leaves above the cotyledons were inoculated with the virus.

Virus inoculation - Mechanical transmission: The inoculum was obtained from leaves of naturally infected basil by grinding in mortar with 0.01 M phosphate buffer (PB) and 0.5% sodium sulfite, pH 7.0. The extract (inoculum) was rubbed with a pistil over the second pair of leaves above the cotyledons of healthy basil plants.

Virus inoculation - Aphid transmission in a non-persistent manner [27]: Laboratory aphids (*Mysus persicae*) were isolated in Petri dishes for 30 min (starvation period) and then fed on naturally virus-infected basil plants for 10 min. Aphids were transferred to healthy basil plants (10 aphids/plant), kept for 10 min and removed.

Virus inoculation - Aphid transmission in a circulative (persistent) manner [27]: The aphids (approx. 100) were transferred from the virus-free stock plants to naturally infected *O. basilicum* and kept for 1 h. After this period, the aphids were transferred to healthy basil plants (10 aphids/plant), kept for 24 h and removed. As a control, the same procedure was performed with aphids kept on healthy plants.

Essential oil extraction and analysis: Essential oils were extracted from aerial part of 5 healthy and 5 virus-infected plants, 20 days after virus transmission. Extraction was made by hydrodistillation in a Clevenger apparatus for 3 h and oils stored at -20°C until analysis. Samples were injected into a GC/MS (Agilent 6890/5975B) employing a DB-5 HT column (30 m x 0.32 mm i.d. x 0.10 μm film). Initial oven temperature was 45°C , increasing at $6^{\circ}\text{C}/\text{min}$ up to 200°C , and then heated at $15^{\circ}\text{C}/\text{min}$ up to 250°C , remaining for 1 min. Injector temperature was 300°C . Helium was used as carrier gas at 1.3 mL/min. The sample injection volume was 1 μL and the split ratio was 50. Source and quadrupole temperatures were 230°C and 150°C , respectively. The electromultiplier voltage was adjusted to 70 eV. Compounds were identified by comparing their mass spectra with data available in the NIST 05 MS library. Oil component proportions were submitted to the *t*-student test at 5% of probability.

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Anexo 6

Nagai, A., Duarte, L.M.L., Chaves, A.L.R., Santos, D.Y.A.C. Does *Potato virus Y* infection affect flavonoid profiles in *Physalis angulata* L.? An in vitro assay. **Brazilian Journal of Botany** (submetido)

Does *Potato virus Y* infection affect flavonoid profiles in *Physalis angulata* L.? An *in vitro* assay.

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Abstract

Physalis angulata L. is an annual herb of Solanaceae. Among several biological activities described for this species, its cytotoxic action is due to the presence of flavonoids. Natural infection of *Physalis angulata* by *Potato virus Y*, strain O (PVY^O), has already been described. The main goal of this study is to verify whether PVY^O infection influences the secondary metabolism of *P. angulata*, evaluating total amount of phenolic compounds and the content and profiles of flavonoids. Twenty days-old plants were distributed into three groups: control (C₁), mechanically injured (C₂) and artificially inoculated (PVY^O). After 21 days, leaves from local and systemic infection were collected and compounds were analyzed by UV-Vis and HPLC. Five flavonoids were partially identified as rutin, kaempferol-4'-*O*-ramnoglucosyl, kaempferol-3-*O*-ramnoglucosyl and two other kaempferol derivatives. Quantitative analysis of total phenol and total flavonoid in the local infection were similar, with higher percentage in the C₁ group, followed by the PVY^O group and, consequently, the lowest percentage present in C₂ group. There were no qualitative differences in flavonoid profiles between healthy and infected groups. However, lower concentrations of these compounds were detected in the systemic infection of infected plants, suggesting that PVY^O influences secondary metabolism by reducing the amounts of flavonoids.

Keywords: Solanaceae, *Physalis angulata*, *Potato virus Y*, phenolic compounds, flavonoids.

Introduction

Physalis angulata L., commonly known as cutleaf groundcherry, wild tomato and winter cherry is an annual, branched and standing herb that belongs to Solanaceae. In Brazil, its popular name is “camapu”, and the species is widely used in traditional medicine to treat bladder and spleen inflammations, rheumatism and earache (Lorenzi 1991).

In Brazil, commercial cultivation of *P. angulata* started in the 1990s, and its edible fruit represents an excellent alternative for small and mid-sized producers (Mairura 2008).

Besides the importance in terms of traditional medicine and food applications, some biological activities have already been described for this plant, such as cytotoxic, antibacterial, anti-inflammatory action, as well as antileishmaniasis actions, which have been associated with some secondary metabolites. The cytotoxic effect is exerted by a flavonoid (myricetin 3-*O*-neohesperidoside) isolated from the methanolic extract of the leaves, while antileishmaniasis and antibacterial effects are carried out by physalins, especially physalin F and physalin B respectively (Ismail and Alam 2001; Choi and Hwang 2003; Silva et al. 2005; Guimarães et al. 2009).

P. angulata has been found to be naturally infected with *Potato virus Y*, strain O (PVY^O) and *Tomato chlorosis virus* (ToCV) in Brazil (Chaves et al. 2010; Fonseca et al. 2013). These pathogens are good examples of limitation factors for crop development.

PVY is the causal agent of important diseases and production losses in solanaceous species crops in Southern America. Weeds from Solanaceae are often potential inoculum sources for infections in tomato and pepper crops. Moreover, numerous species of *Physalis* are described as potential reservoirs of the virus in Southern and Northern America (Kerlan and Moury 2008; Eiras et al. 2012). This virus is considered to be strongly immunogenic and isolates have historically been divided into three main strains: Ordinary (O), Necrotic (N) and Chlorotic (C). PVY^O and PVY^C are distinguished based on hypersensitive reaction in potato cultivars bearing the resistance genes. PVY^N differs from PVY^O and PVY^C in causing a veinal necrosis reaction in *N. tabacum* cv. Samsun or cv. Xanthi (Shukla et al. 1994). Two main variants have emerged in the past two decades: PVY^{NTN}, characterized by its ability to induce tuber necrosis, and PVY^{NW}, which differs in its pathogenicity and in the presence of serotype O-C instead of serotype N (Kerlan and Moury 2008).

The exposure to pathogens can alter the abundance and/or composition of secondary metabolites in plants, which are reputedly responsible for the pharmacological activity of medicinal plants, as described above (Bruni et al. 2005). These metabolic changes can still be different in the local of infection or systemically (Duarte et al. 2008).

There are no works concerning viral influence on the secondary metabolism of *P. angulata*. In this scenario, the present study aimed to assess the influence of induced infection by PVY^O on phenolic metabolites of this plant.

Material and methods

Plant material

Seeds of *Physalis angulata* L. were obtained from commercial fruits. They were sown in sterilized land, irrigated daily and kept in greenhouse. Plants with two or three leaves above the cotyledonaries were transplanted to pots. A voucher was deposited at the Herbarium of the Biosciences Institute from University of São Paulo – SPF (Santos 6).

Virus inoculation - Mechanical transmission

The virus used to infect plants was the *Potato virus Y* – ordinary strain (PVY^O) – isolated from naturally infected *P. angulata* collected in Mairiporã – SP and belonging to the plant virus collection of the Plant Virology Laboratory in Biological Institute, São Paulo (71/2009).

The inoculum was prepared from leaves of infected *P. angulata* crushed in a mortar with inoculation buffer (0.01M phosphate buffer, pH 7.0, with 0.04M sodium sulfite) following the ratio of 1/3 (weight/volume – w/v). Three-month-old plants of *P. angulata* were experimentally inoculated with 50µL of virus inoculum on the third and fourth leaves above the cotyledonaries, previously sprinkled with 400 mesh carborundum abrasive.

Determination of the highest viral concentration in *P. angulata*

About 20 days after germination, *P. angulata* seedlings were experimentally inoculated as described. The leaves inoculated and those immediately above were separately collected 7, 14, 21 and 28 days after inoculation (DAI). Each DAI group was formed by three individuals.

The leaves were weighted and crushed in a plastic bag with extraction buffer (phosphate buffer saline with Tween 20 (PBST) + 2% polivinilpirrolidone (PVP)) according to the ratio of 1/10 (w/v).

The extracts were submitted to the serological test Double Antibody Sandwich - Enzyme Linked Immuno Sorbent Assay (DAS-ELISA) (Almeida et al. 2010). Briefly, 50µL of the antiserum against PVY^O (AGDIA) diluted in carbonate buffer, pH 7.4 (1/200 – volume/volume - v/v) were applied in the wells of the polystyrene plate, and kept at 37°C for 2 h. The plate was washed 3 times with 0.1M PBS + 0.5% “Tween” 20 (PBST), and 50µL of the plant extracts were added. After incubation at 37°C for 2 h, the plate was washed and 50µL of anti-immunoglobulin conjugated to the phosphatase alkaline enzyme diluted in PBST + 2% PVP + 2% BSA (bovine serum albumin) was added to the plate. The addition of 50µL of p-nitrophenyl phosphate dissolved in substrate buffer 1/1 (w/v) completed the test. The reaction intensity was measured by absorbance readings (A405nm) in an ELISA reader (Bio Rad, model 3550-UV).

Experimental design

The experiment consisted of three groups (Control 1 – C₁, Control 2 – C₂ and Virus infected – PVY^O) with 50 plants each. These plants were randomly divided into five repetitions (R1, R2, R3, R4 and R5) with 10 plants each. The inoculated leaves (L) and non-inoculated leaves (S) of all individuals were collected separately, 21 days after inoculation. This time was selected after determining the highest virus concentration by DAS-ELISA (Almeida et al. 2010) with PVY^O antiserum. The samples were formed pooling material of the 10 plants.

The C₁ group was not submitted to any treatment. The fourth and fifth leaves above the cotyledonaries of the C₂ group were rubbed with 50µL of inoculation buffer. Leaves of PVY^O group at the same position as those from C₂ group were treated with 50µL of the virus inoculum (Duarte et al. 2008).

Phenolic compounds extraction and quantification

Crude extracts were obtained from powdered and dried leaves of *P. angulata* under reflux with 80% MeOH for 1h. This procedure was repeated three times. Extracts were filtered, pooled, concentrated in a rotary evaporator, washed with toluene and resuspended in MeOH.

Total phenol was analyzed by the Folin-Ciocalteu method, using 0.1mL of the sample, 3.9mL of distilled water, 0.75mL of concentrated solution of sodium carbonate and 0.25mL of Folin-Ciocalteu reagent. After 2h the solution was analyzed in a spectrophotometer (UV - 1650 PC – Shimadzu) at 760nm (Waterman and Mole 1994). A calibration curve using *p*-coumaric acid was constructed for quantification.

Flavonoid content was analyzed by adding 0.5mL of aluminium chloride methanol solution to 0.5mL sample extract. This solution was stored for 15 min and then it was analyzed in a spectrophotometer (UV - 1650 PC – Shimadzu) at 420nm. In this case, the calibration curve was constructed with rutin.

High pressure liquid chromatography (HPLC) analysis

A 50µL aliquot of methanol extract was injected in a Agilent HP series II 1090 – DAD device with a reverse phase column Zorbax C18 (4.6 x 250 mm, 5 µm). The mobile phase was composed of 0.1% acetic acid (A) and acetonitrile (B) with the following gradient: 0-5 min (12% B); 5-8 min (12% up to 20% B); 8-20 min (20% up to 30% B); 20-28 min (30% up to 35% B); 28-38 min (35% up to 50% B); 38-48 min (50% up to 65% B) and 48-50 min (65% up to 100% B), isocratic for 5 min and 55-60 min decreasing up to 12%. The flow was 0.5mL.min⁻¹ (0 – 50 min), 1mL.min⁻¹ (50 – 55 min) and 0.5mL.min⁻¹ (55 – 60 min). The column temperature was 40°C and the chromatograms were processed at 352nm (Furlan et al. 2010).

Standard solutions with distinct concentrations of rutin were analyzed by HPLC to construct a standard curve used for individually flavonoid quantification.

Flavonoid isolation and identification

Larger amounts of crude extract were obtained from healthy plants specially cultivated for this purpose.

The flavonoids were isolated by glass column chromatography using polyvinylpyrrolidone (PVPP) and MeOH as stationary and mobile phases, respectively, combined with semi-preparative HPLC (Agilent 1200) analysis. The column used in the semi-prep HPLC was a Zorbax C18 (9.4 x 250mm, 5 μ m) and the mobile phase was the same described above, using pure water instead of 0.1% acetic acid in constant flux of 4mL.min⁻¹, and detection at 352nm.

The isolated flavonoids were identified following standard procedures by UV-VIS absorption spectroscopy (240–500nm), using methanol solutions and addition of shift reagents, acid and enzymatic hydrolyses, cellulose TLC and comparison with authentic samples (Mabry et al. 1970; Markham 1982).

Statistical analysis

Values in the figures and table are mean values of five independent replicates. Significant differences among treatments were analyzed by one-way ANOVA followed by a post-hoc comparison using Tukey's test and by Kruskal-Wallis test followed by a post-hoc comparison according to Giraudox 2011, using R (R version 3.0.2 (2013-09-25)) for windows.

Results and Discussion

Both local and systemic infections showed higher virus concentration after 7 days after inoculation (DAI). Although the virus concentration at the local infection on the 28th DAI was not statistically different from 14th and 21st DAI, a slight drop was observed (Table 1). Considering the systemic infection, the absorbance values were lower only for the 7th DAI samples. Based on these results, the 21st DAI was chosen as the best period for harvesting leaves for phenolic bioassay, avoiding the slight decrease due to local infection in an effort to obtain larger biomass.

Significant differences between total phenol concentrations in the three treatments in the local infection were recorded. The C₁ group showed the highest concentration of total phenol, while the C₂ group presented the lowest concentration. Comparing the C₁ to the other groups, it is possible to say that mechanical wound reduced the total phenol amount. An increase in the total phenol concentration was observed in the PVY^O compared to the C₂ group (Fig. 1a), possibly due to the presence of the virus. This increase, however, did not reach the basal total phenol amount found in C₁ group, probably, because molecules from the virus, called effectors, triggered a response caused by successful pathogens known as effector-triggered susceptibility (ETS). The increase in the total phenol concentration in the PVY^O group could be higher compared to the C₁ group whether these effectors were recognized by proteins codified by R genes, activating an accelerated

and amplified response called effector-triggered immunity (ETI); however, R gene related to PVY⁰ resistance has not been described in *P. angulata* (Jones and Dangl 2006).

This response was not similar to what was observed for *Datura stramonium* L. infected by *Potato virus X* (PVX), which showed a decrease in the content of total phenolic compounds in the infected plants on the 10th DAI compared to untreated plants. Moreover, in the *D. stramonium*-PVX pathosystem, no significant differences between the control and the mechanically damaged group were found. In plants of *Nicotiana tabacum* cv. Xanthi infected with *Tobacco mosaic virus* (TMV), an increase in the content of total phenol in the inoculated leaves up to the 6th day was observed, with a subsequent decrease in this content, below the level exhibited by the control leaves. Therefore, by analyzing the results obtained for *P. angulata* and comparing them with the works mentioned above, it seems that the content of total phenols in the infected leaves depends on the time leaves are collected (Tanguy and Martin 1972; Duarte et al. 2008).

Although the general response seems the same for local and systemic infection, no significant differences were observed in the systemic infection, when the C₁ group is compared to the PVY⁰ group. These groups were different from the C₂ group, which showed lower total phenol content (Fig. 1a). This disagrees with a previous study about the *D. stramonium*-PVX pathosystem, which showed a decrease in the content of total phenolic compounds in the infected plants, compared to the control (Duarte et al. 2008). These differences in the results are not unexpected, since PVX and PVY have different replication strategies. PVX genome contains five open reading frame (ORF), encoding replicase, three putative protein components called triple gene block and the coat protein. On the other hand, PVY genome comprises a single ORF coding for a polyprotein that generates mature products after ongoing an autoproteolytic processing cascade (Kerlan and Moury 2008).

The response observed for total flavonoids was similar to the total phenol content in the local and systemic infections (Fig. 1b). The mechanically injured group (C₂) always presented the lowest values. This response suggests that mechanical damage influenced the secondary metabolism by reducing the phenolic substances quantities. Also, this reduction was even higher, when compared to the pathogen presence.

Defense mechanisms used for wounding in plants overlap those involved in pathogen attack. It has been demonstrated that wounding up-regulates some transcription factors that positively regulates secondary metabolism (Cheong et al. 2002). Therefore, it was supposed the content of phenolic compounds in the C₂ group did not differ from the PVY⁰ group. It would be necessary to survey global gene expression pattern after wounding and compare it with gene expression pattern following pathogen attack to better understand the differences in the response observed for C₂ and PVY⁰ group.

The HPLC analysis revealed the presence of five different flavonoids in leaves of *P. angulata*, and no qualitative difference between the treatments.

Three flavonoids were isolated, in sufficient amounts, to afford their identification as rutin, kaempferol-4'-*O*-ramnoglucosyl and kaempferol-3-*O*-ramnoglucosyl. The two other flavonoids were not completely identified, but the UV data suggests they are kaempferol derivatives.

In the local infection, no significant differences were observed, when comparing the amounts of each flavonoid between treatments (Fig. 2a). On the other hand, a significant decrease in the amount of the five flavonoids in the systemic infection, comparing the control group C₁ to the PVY^O group was found (Fig 2b).

Comparing these data to the literature, the response obtained for *P. angulata* was distinct from that observed for *Vitis vinifera* L. infected with *Grapewine leaf-roll-associated virus 3* (GLRaV-3). Higher content of flavonols in the infected fruits of *V. vinifera* was found. Nonetheless, the content of anthocyanin has decreased. The authors suggested that the decrease in the expression of the main genes involved in the flavonoid biosynthetic pathway could explain this variation (Vega et al. 2011). Further investigations on gene expression level in the infected plants of *P. angulata* should be conducted to better explain our results.

The analysis of three cultivars of *Solanum tuberosum* infected with *Potato virus Y* (PVY^{NTN}) showed a decrease in rutin levels for two of them (*S. tuberosum* cv. Igor and *S. tuberosum* cv. Desirée). This decrease was larger for *S. tuberosum* cv. Igor, which is considered susceptible to the virus and presents severe symptoms after infection. *S. tuberosum* cv. Desirée, with a slight decrease in rutin levels, did not display severe symptoms, being considered a tolerant cultivar. Notwithstanding, for *S. tuberosum* cv. Sante, considered resistant to PVY^{NTN} infection, there was a small increase in the rutin content (Kreft et al. 1999). It seems that the decrease in the flavonoid content is related to the susceptibility to the virus.

Analyzing *P. angulata*, this species could be suggested as tolerant, but not resistant, to PVY^O, since no severe symptoms were observed (data not shown) and a decrease in the content of the flavonoids in the systemic infection was found, corroborating the hypothesis suggested above. Two pathways to explain this decrease have been suggested: (a) flavonoids have an active role in the defense mechanism of the plant, and then they become less available in plant; or (b) the synthesis of these flavonoids seems to be biosynthetically redirected to the production of other phenolic substances (Kreft et al. 1999). More detailed studies are needed, including gene expression investigations, to help solve this challenge.

Plant response to fungi infections is different from that observed with viruses. In general, there is an increase in the content of phenolic compounds in plants infected with fungi. For *Malus* spp. infected with *Venturia inaequalis* (Cke.) Wint, an increase in the flavonols content, such as rutin and quercitrin, was observed. In plants of corn (*Zea mays* L.) inoculated with *Colletotrichum graminicola* (Ces.) and *Helminthosporium maydis* Nisik and Miy, a significant increase in the content of two phenolic compounds was observed, whose concentrations in healthy plants were low (Lyons et al. 1990; Petkovšek et al. 2009).

Several studies on fungal infections in plants have been published, indicating that these plants have a higher content of certain phenolic compounds, following a response pattern. In turn, in plants infected with virus, a general response in the content of phenolic compounds is not observed, possibly because only a few works have addressed this pathosystem, or because that virus does not trigger similar responses on the plants metabolism.

Interaction PVY^O/*P. angulata* and his interference on the phenolic metabolites is important, because current knowledge indicates that PVY was present in pre-Columbian America and might have followed different evolutionary pathways as a result of co-evolution with various solanaceous hosts (Glais et al. 2002).

These results reveal no qualitative variation in the flavonoids profile. On the other hand, a quantitative variation in flavonoid contents was observed. The response patterns of total phenol, and total flavonoid in the local infection were similar, with higher percentage in the C₁ group, followed by the PVY^O group and, consequently, the lowest percentage present in C₂ group. The isolated flavonoids showed no significant differences.

For the systemic infection, concerning total phenol and total flavonoid, no differences between C₁ and PVY^O group were observed (Fig. 1a, b). However, a reduction in their content was detected in C₂ group. The isolated flavonoids showed a different pattern, with a significant decrease in the content of these substances in the PVY^O group. This kind of response is not similar to that obtained in the infection caused by fungi, which shows an increase in the content of phenolic compounds.

Cytotoxic activity and other biological activities have already been described for *P. angulata* associated with flavonoid contents. Thus, the viral infection may produce an undesirable effect in this species, since a decrease in these metabolites occurs. So, the results presented in this work underlines the importance of the phytosanitary control of camapu plants used in the preparation of extracts, or in the isolation of substances with biological importance.

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Table 1. Mean ($\pm$ SD) of absorbance value in DAS-ELISA assay with leaf extracts from infected plants of *P. angulata*.

DAI ^a	Infection	
	Local	systemic
7	1.490 $\pm$ 0.121 ^a	0.864 $\pm$ 0.068 ^c
14	2.016 $\pm$ 0.126 ^b	1.744 $\pm$ 0.093 ^d
21	1.996 $\pm$ 0.096 ^b	1.760 $\pm$ 0.044 ^d
28	1.887 $\pm$ 0.106 ^{ab}	1.682 $\pm$ 0.129 ^d

^a Days after inoculation. Different letters represent significant differences between the treatments in the same infection

Figure captions

Fig. 1 Total phenolics and flavonoids in leaves of health and PVY-infected plants of *Physalis angulata*. Numbers above bars correspond to mean $\pm$ SD. Different letters represent significant differences between treatments in the same kind of infection. **a.** Total phenolic compounds (mg phenol/mg d. wt) - local infection: $p < 0.0001$; systemic infection: $p = 0.0014$. **b.** Total flavonoids (mg flav./mg d. wt) - local infection: $p = 0.0039$; systemic infection: $p = 0.0044$

Fig. 2 Amount of each flavonoid identified in leaves of *Physalis angulata* (mg/mg plant). **a.** Local infection. **b.** Systemic infection. Different letters represent significant differences between treatments

Anexo 7

Tomomitsu, A.T., Chaves, A.L.R., Duarte, L.M.L., Eiras, M., **Santos, D.Y.A.C.** 2014. Effect of *Cowpea aphid-borne mosaic virus* on growth and quantitative variation of total phenolics and flavonoids from *Passiflora edulis* Sims. **Boletim de Botânica da Universidade de São Paulo** 32: 141-144.

EFFECT OF COWPEA APHID-BORNE MOSAIC VIRUS ON GROWTH AND QUANTITATIVE VARIATION OF TOTAL PHENOLICS AND FLAVONOIDS FROM *PASSIFLORA EDULIS* SIMS

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Abstract - (Effect of *Cowpea aphid-borne mosaic virus* on growth and quantitative variation of total phenolics and flavonoid from *Passiflora edulis* Sims.) *Cowpea aphid-borne mosaic virus* induces woodiness of fruit pericarp, stunting, leaf mosaic and blistering in passion fruit plants. Total amount of phenols and flavonoids from leaves of healthy and artificially infected plants were quantified by the Folin-Ciocalteu method and reaction with aluminum chloride. Heights of all plants were measured at the beginning and end of the experiment. Infected plants presented 80% less height growth than healthy plants. There was no statistical difference in the amount of total phenols and flavonoids among treatments.

Key words: *Potyvirus*, passion fruit woodiness disease, secondary metabolism.

Resumo - (Efeito do *Cowpea aphid-borne mosaic virus* sobre crescimento e variação quantitativa de fenois totais e flavonoides de *Passiflora edulis* Sims.) *Cowpea aphid-borne mosaic virus* induz o endurecimento do pericarpo do fruto, nanismo, mosaico foliar e bolhas em plantas de maracujá. Os teores totais de fenois e flavonoides de folhas sadias e artificialmente infectadas foram quantificados pelo método de Folin-Ciocalteu e reação com cloreto de alumínio. Alturas de todas as plantas foram medidas no início e no final do experimento. As plantas infectadas apresentaram alturas 80% menores do que as plantas sadias. Não houve diferença estatística nos teores de fenois totais e de flavonoides entre tratamentos.

Palavras-chave: *Potyvirus*, endurecimento dos frutos do maracujazeiro, metabolismo secundário, fenois, flavonoides.

Introduction

Passifloraceae consists of circa 20 genera and 600 species distributed across hot climate regions, such as the Americas and Asia (Souza & Lorenzi 2005). *Passiflora* is the prevailing genus, with approximately 520 species distributed mainly in tropical and subtropical regions, 150 of which are native to Brazil (Cervi 2005). In this country, *Passiflora* species are popularly known as *maracujá*, an indigenous word meaning "fruit to suck to" or "gourd-shaped fruit" (Teixeira 1994). Passion fruit plants (*Passiflora* spp.) are climbing, sub woody vines that produce grape-shaped fruits (Cunha *et al.* 2004).

Brazil is the world's largest producer of the yellow passion fruit (*Passiflora edulis* Sims.), with a cultivated area of around 62,019 ha (Agrianual 2013). Within the country, the northeast region has the largest production figures (Meletti 2011). Only two passion fruit species are commercially important, the yellow passion fruit (sometimes called sour passion fruit), which is used in the juice industry, and *P. alata* Curtis (the sweet passion fruit), destined for *in natura* consumption. Known in popular medicine for their pharmacological properties for which their secondary metabolites are responsible, passion fruit varieties are considered as functional foods. These fruit present antioxidant and medicinal properties, and are used in

the treatment of anxiety and irritability (Nodari *et al.* 2000, Dhawan *et al.* 2004). Patel (2009) described the antihypertensive and antioxidant action of a *P. edulis* methanolic fraction, which was shown to contain polyphenols, while Zeraik *et al.* (2010) reported that the antioxidant action of these fruit is due to the presence of flavonoids. However, commercial passion fruit cultures are likely to acquire a series of different diseases, mainly caused by viruses (Anjos *et al.* 2001, Chagas & Colariccio 2006, Fisher & Rezende 2008).

Passion fruit woodiness (PFW), the most important disease to affect passion fruit plantations in Brazil, is caused by the *Cowpea aphid-borne mosaic virus* (CABMV, *Potyvirus*). This viral infection causes hardening of the pericarp, a downside that lowers fruit quality, reducing marketable production numbers and leading to economic losses (Peruch *et al.* 2009). Other systemic symptoms such as mosaic and blistering are also associated to PFW (Bock & Conti 1974).

In spite of this, the literature lacks scientific papers describing the influence of CABMV in the metabolism of phenols and flavonoids present in the passion fruit. In this scenario, the present study aimed to assess the role of CABMV in the growth of the yellow passion fruit, and its effect on the levels of total phenols and flavonoids, by comparing extracts obtained from healthy and CABMV-infected leaves.

Material and methods

The CABMV isolate was obtained from *P. edulis* (yellow passion fruit) produced in Monte Alegre do Sul, state of São Paulo, Brazil, and characterized according to Silva *et al.* (2012). Sample leaves, collected in the field and stored in calcium chloride at -20°C, were triturated with previously sterilized and chilled mortar and pestle containing a 1:5 (g:mL) sodium sulfite 0.5% medium (pH 6.0) (Gibbs & Harrison 1976) to obtain the viral inoculum. Fifty-four yellow passion fruit plants grown from seeds (IAC-277) sowed in previously sterilized soil and kept in a greenhouse were used in experiments to assess growth and variation in total phenol and flavonoid contents. Ninety days after germination, the plants were randomized into three treatment groups: plants rubbed on with a sodium sulfite 0.5% solution (L1); plants inoculated with CABMV + sodium sulfite 0.5% solution (L2); and a control group (C). Mechanical infection of CABMV onto L2 plants was carried out rubbing the viral inoculum on the adaxial epidermis of the third leaf above the cotyledonary node axil. L1 plants had the same leaves rubbed, but with the buffer solution only. Control plants were not exposed to any treatment. Within each group of 18 plants, three randomized repeats ($n = 3$) were carried out. The virus was detected by PTA-ELISA using a CABMV-specific antiserum.

Growth was assessed measuring plant height of each individual prior to and after inoculation. Quantification of total phenols and flavonoids was

conducted 30 days after inoculation using 2.0 g of dried leaves. Powdered leaves were macerated under heating in MeOH 80% reflux for 1 h. The extraction procedure was repeated three times. Extracts were filtered, pooled, concentrated in a rotatory evaporator, washed in toluene and resuspended in MeOH 100% (according to Furlan *et al.* 2010, with modifications). Total phenols were quantified according to the Folin-Ciocalteu method (Waterman & Mole 1994), while flavonoids were measured by reaction with aluminum chloride (adapted from Motta *et al.* 2005) using *p*-coumaric acid and quercetin as reference, respectively.

Mean contents of total phenols and flavonoids were evaluated in ANOVA and compared using the Tukey test ($\alpha = 5\%$).

Results and discussion

PTA-ELISA confirmed that groups C and L1 plants were healthy, while L2 plants were infected by CABMV. Apart from the positive results obtained in the assay, L2 plants exhibited typical visible symptoms such as mosaic and blistering on leaves (Figure 1). This viral infection influenced growth of L2 plants, whose mean growth values were 80% lower, in comparison with C and L1 (Table 1). Since mean height of plants challenged with buffer (L1) did not differ from the height of control individuals, it is possible to conclude that this decrease in height was indeed caused by CABMV infection.

Fig. 1: (A) Mosaic and blistering (arrows) in *Passiflora edulis* Sims. infected with CABMV. (B) Healthy *Passiflora edulis* Sims. (Photo credits: Marcelo Eiras.)

Pathogens like viruses, phytoplasmas and nematodes induce symptoms such as chlorosis and dwarfism, and therefore directly affect photosynthesis and development of the host plant (Agrios 2005). These symptoms are seen as phenotypical changes in cell physiology and structure associated with impaired growth and development (Maule *et al.* 2002). Agrios (2005) and Hull (2009) observed that lower growth is the main symptom prompted by viruses in plants, while

Fisher & Rezende (2008) added that, apart from CABMV, the *Passion fruit woodiness virus* (PWV, *Potyvirus*) also induces lower growth and development of passion fruit species.

In spite of the marked effect of viral infections on passion fruit development, no statistically significant difference was observed in total phenol and flavonoid contents across treatments in the present study (Table 1).

Table 1: Total phenols and flavonoids levels ($\mu\text{g}/\text{mg}$ dry leaf) in methanolic extracts of *Passiflora edulis* Sims.

Treatment	Height ^{1,4}	Total phenols ^{2,4}	Total flavonoids ^{3,4}
C	25.833 ^a $\pm$ 17.5744	25.233 ^a $\pm$ 8.708	1.320 ^b $\pm$ 0.382
L1	24.056 ^a $\pm$ 15.2937	26.328 ^a $\pm$ 5.572	1.332 ^b $\pm$ 0.170
L2	5.3333 ^b $\pm$ 6.167	33.413 ^a $\pm$ 7.223	0.992 ^b $\pm$ 0.169

Obs.: Mean $\pm$ SD difference in initial vs. final height (cm) of plants challenged with each treatment. C: control; L1: sodium sulfite 0.5%; L2: challenge with CABMV + sodium sulfite 0.5%. 1- cm; 2- μg E *p*-cumaric acid/mg dry leaf; 3- μg E quercetin/mg dry leaf; 4- Values followed by identical letters do not present statistically significant difference ($\alpha = 0.5\%$).

Increased total phenol contents have clearly been shown to be a response produced by plants infected with fungi (Vidhyasekaran 2004, Agrios 2005). The phenolic content, from methanol extratcts of *Passiflora nitida* Kunth and *P. foetida* L. were shown to have antimicrobial activity against *Escherichia coli* by agar diffusion and turbidity assays (Bendini *et al.* 2006). However, the actual influence of a viral infection on phenol levels has yet to be fully investigated. Ajmal *et al.* (2011) analyzed *Gossypium* spp. infected with Cotton leaf curl virus (CLCuV, *Begomovirus*) and observed a decrease in phenol contents of leaves. Similarly, a drop in phenol levels was reported for directly infected (local infection) leaves of *Datura stramonium* L. challenged with *Potato virus X* (PVX, *Potexvirus*) by Duarte *et al.* (2008). However, the authors also observed that levels of these metabolites were higher in leaves challenged only with phosphate buffer and in leaves above the challenged ones, possibly induced by mechanical injury. The results obtained in the present study for the described passion fruit CABMV system do not confirm the premise that mechanical injury and/or viral infection induce some change in total phenol contents in passion fruit plants.

Considering flavonoid levels, few studies have proved the influence of a virus on levels of these metabolites. As the results herein reveal, CABMV infection or mechanical injury *per se* did not affect total flavonoid contents in yellow passion fruit plants (Table 1). Nevertheless, the literature has produced diverse results as to the effect of viruses on levels of this class of compounds. Kreft *et al.* (1999) report a decrease in rutin levels, a well-known flavonoid, in *Solanum tuberosum* L. susceptible to *Potato virus Y NTN* (PVY^{NTN}, *Potyvirus*). However, in an elegant study that shed new light on the effect of the Grapevine leaf-roll-associated virus-3 (GLRaV-3, *Ampelovirus*) on *Vitis*

vinifera L. var. Cabernet Sauvignon, Vega *et al.* (2011) found that flavonoid levels first increased, only to drop afterwards, during the ripening period of fruits. Kreft *et al.* (1999) and Vega *et al.* (2011) did not identify any qualitative difference in flavonoids profiles of plants infected with viruses and healthy individuals.

Phenolic compounds such as flavonoids are believed to play a role in a plant's defense against the attack by pathogens (Croteau *et al.* 2000), and their levels might be higher in infected plants, compared with healthy individuals. In spite of the fact that CABMV significantly changes the growth of *P. edulis*, this effect is not observed in secondary metabolism, which involves the action of flavonoids and diverse phenolic substances, as well as other compounds (Agrios 2005, Dewick 2009).

Conclusion

In spite of the fact that CABMV changes the growth of *P. edulis*, leading to a marked decrease in plant height, no statistically significant differences were observe in total phenol and flavonoid levels between healthy and experimentally challenged yellow passion fruit plants.

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Anexo 8

Myiashira, C.H., Tanigushi, D.G., Gugliotta, A., Santos, D.Y.A.C. 2010. Comparison of radial growth rate of the mutualistic fungus of *Atta sexdens rubropilosa* Forel in two culture media. **Brazilian Journal of Microbiology** 41: 506-511.

COMPARISON OF RADIAL GROWTH RATE OF THE MUTUALISTIC FUNGUS OF *ATTA SEXDENS* *RUBROPILOSA* FOREL IN TWO CULTURE MEDIA

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ABSTRACT

In vitro culture of the mutualistic fungus of leaf-cutting ants is troublesome due to its low growth rate, which leads to storage problems and contaminants accumulation. This paper aims at comparing the radial growth rate of the mutualistic fungus of *Atta sexdens rubropilosa* Forel in two different culture media (Pagnocca B and MEA LP). Although total MEA LP radial growth was greater all along the bioassay, no significant difference was detected between growth efficiencies of the two media. Previous evidences of low growth rate for this fungus were confirmed. Since these data cannot point greater efficiency of one culture medium over the other, MEA LP medium is indicated for *in vitro* studies with this mutualistic fungus due its simpler composition and translucent color, making the analysis easier.

Key words: leaf-cutting ants, mutualistic fungus growth, mycelial growth, *Leucoagaricus*, *Atta sexdens*

INTRODUCTION

Fungus-growing ants are distributed only in the New World and belong to the monophyletic tribe Attini (Hymenoptera-Formicidae-Myrmicinae), which is composed of 12 genera and approximately 210 species.

Among all attine, usually referred to as fungus-growers, the two most phylogenetically derived genera, *Acromyrmex* and *Atta*, are more commonly known as leaf-cutting ants (8). The leaf-cutting ants are an important forest herbivore, exploring a great variety of plants which are used to cultivate a specific basidiomycete fungus (6). Several authors have identified this microorganism as *Leucoagaricus gongylophorus*

(=*Leucocoprinus gongylophorus*) based on the morphology of fruit-bodies which grow inside *Atta sexdens rubropilosa* (2) and *Atta cephalotes* (10) nests, or using molecular sequences (25). This fungus produces a special mycelia structure called gongylidia, rich in glycogen and used as food for the leaf-cutting larvae (1).

Leaf-cutting ants and the basidiomycete fungus possess an intrinsic mutualistic relationship, strongly integrated with antibiotic, nutritional and physiological co-dependence. Leaf-cutting ants protect the mutualistic fungus from parasites and potentials competitors (7, 8), while the fungus is an essential food source for the larvae and queen (17, 20). Other ants in the nests have plant sap as an important food source (23).

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Leaf-cutting ants have been considered a dangerous herbivore to some important crop fields. Several methods have already been proposed for the control of these insects. Since all of them present some environmental disadvantage, continuous search for an effective and less pollutant control method acting directly on ants or on fungal development is the focus of much research (14, 21, 22).

Studies using mutualistic fungi are difficult due to the very slow fungal growth in culture media (15, 16, 21, 22). For example, the mutualistic fungus of *Atta sexdens priventris* reached 57 mm in diameter after 63 days of experiment with an organic medium called Pagnocca A; 34,4 mm with the medium called V8 juice agar; 46,2 mm with an organic medium called Celulose-asparigine; and 16,8 mm with the mineral medium called Murashige & Scoog (15). Such slow growth rates turn the storage very difficult, and the fungus culture is often impregnated with contaminants.

In this paper, two solid culture media, MEA LP and Pagnocca B, were evaluated for the efficiency of *in vitro* fungal growth promotion. MEA LP culture medium was prepared by combination of two traditional culture media (MEA with yeast addition and MEA peptone). Pagnocca B was first described by Silva-Pinhati *et al.* (25) for bioassays with the mutualistic fungus of *Atta sexdens* and consists of a new buffered supplemented formulation of Pagnocca Medium A.

MATERIAL AND METHODS

Eight leaf-cutting ant nests maintained in laboratory were used as fungi source. Fungi fragments and some ants were removed from each nest and transferred to sterile pots, previously autoclaved at 120°C and 1.1 atm (eight fungi matrix). The transport of some ants was crucial for successfully fungi culture, because they are able to clean fungi fragments and stimulate its growth. Small isolated mycelium portions of each matrix were inoculated on Petri dishes containing MEA LP, using a sterile laminar flux chamber, for development of the initials cultures.

Two culture media compositions were tested. The first is a common fungi culture medium containing malt extract

combined with yeast and peptone (MEA LP – 20 g malt extract, 5 g bacteriological peptone, 2 g yeast extract, 20 g agar, distilled water up to 1 L). The other culture medium, called Pagnocca B by Silva-Pinhati *et al.* (26), is composed of 10 g glucose, 2 g sodium chloride, 2 g bacteriological peptone, 10 g malt extract, 17 g agar, 20 g casein hydrolysate, 20 g soybean flakes, 20 g oat flakes, 3.8 g sodium phosphate, 2.5 g citric acid, distilled water up to 1 L, and pH adjusted to 5.0. Both MEA LP and Pagnocca B culture media were autoclaved at 120 °C and 1.1 atm for 30 min.

Sterile Petri dishes were prepared with 15 mL of culture medium, and kept in a sterile laminar flow chamber under UV light until culture medium solidification. Five millimeter discs containing the mutualistic fungus from initial cultures were transferred to test Petri dishes and placed in the center. Eight Petri dishes, each corresponding to one fungi matrix, were prepared for both MEA LP and Pagnocca B media. The dishes were incubated in a B.O.D. chamber in the dark at 25 °C ($\pm 1^\circ\text{C}$).

Two perpendicular straight lines were drawn on the bottom of each Petri dish. The crossing point coincided with the center of the 5 mm initial fungi disc. Radial growth measurements were recorded weekly from the edge of the initial inoculum until the extreme area of fungi mycelia development, following the four segments formed by the two perpendicular lines (Figure 1). Data for each week correspond to means of four measurements, each one carried out with one segment.

Bioassays were ended when the fungi mycelia reached the Petri dish wall in any dish. Daily fungal growth rate was calculated for each fungi matrix, and expressed as $\text{mm}\cdot\text{day}^{-1}$. Student's T test was used to evaluate differences significance of fungal growth rates between the two culture media.

RESULT AND DISCUSSION

Previous bioassays with traditional *in vitro* culture medium suggested that MEA LP is more effective regarding the growth promotion of the mutualistic fungus of *Atta sexdens* development, in comparison with MEA+yeast (MEA LP

without peptone) or BDA+yeast (10 g dextrose, 20 g agar, juice from 200 g potato cooked in 500 mL water, distilled water up to 1 L) (data not shown). Pagnocca *et al.* (21, 22) and Godoy *et al.* (11) used a growth medium very similar to MEA LP used at the present research, containing glucose, sodium chloride, bacto-peptona, malt extract and agar. Silva-Pinhata *et al.* (26) suggested a new growth medium, called Pagnocca B, as the best option for this mutualistic fungus culture. In the present paper, both media MEA LP and Pagnocca B are compared for growth efficiency of *in vitro* culture of this fungus species. The observation of gongylidia on mycelia fragments by optical microscopy supports the identity of the mutualistic fungus (*Leucoagaricus* sp) in the *in vitro* cultures (Figure 2) (6, 13, 27).

Mean values of total radial growth of mutualistic fungus related to time are presented in Figure 3. Bioassays completion took seven weeks. All MEA LP measurements showed higher values than those of Pagnocca B medium, which were more evident after the 28th day. However, no significant differences ($p \leq 0.05$) were observed of *in vitro* radial growth of this fungus species. Although the MEA LP values were always higher than those obtained with Pagnocca B, the available data failed to support the hypothesis of better growth promotion of one medium over the other.

Figure 1. Petri dish used for fungus growth bioassay. Black arrow indicates the edge of initial inoculum. White arrow indicates the edge of fungi radial growth six weeks after bioassay start. Letters A, B, C, D correspond to the four segments used for growth measurements.

Figure 2. Mycelia detail from mutualistic fungi of *Atta sexdens rubropilosa*. Enlarged apical structures correspond to gongylidia (arrows). Bars = 40 μ m. (Photo: A. M. Gugliotta).

Figure 3. Means and confidence intervals of *in vitro* radial growth of mutualistic fungus of *Atta sexdens rubropilosa* in MEA LP medium and Pagnocca B medium (n = 8). Same letter over bars indicates that there is no significant difference by using Student T test ($P < 0.05$).

Based on total radial growth, daily growth rate was calculated for each one of the eight fungus matrices (Table 1). Daily growth rates among MEA LP samples were less variable, in comparison with Pagnocca samples. The standard deviation calculated using the eight MEA LP dish plates was very low. Only one sample presented daily growth rate lower than 0.4 mm.day⁻¹ with MEA LP medium (matrix 6 = 0.380 mm.day⁻¹). All other samples presented values between 0.426 mm.day⁻¹ and 0.684 mm.day⁻¹. Mean daily growth rate for samples cultivated with Pagnocca B medium was lower than the value obtained for MEA LP, 0.440 mm.day⁻¹ and 0.515 mm.day⁻¹, respectively. Four of the eight sample matrices presented growth rate lower than 0.4 mm.day⁻¹ with Pagnocca B medium. Comparing MEA LP and Pagnocca B values for the same matrix, four of them presented higher MEA LP values (matrices 4, 5, 7 and 8), while the others (matrices 1, 2, 3 and 6) grew better with Pagnocca B. Matrix 8 presented, at the same time, the highest growth rate with MEA LP (0.684 mm.day⁻¹) and the lowest one with Pagnocca medium (0.316mm.day⁻¹).

Table 1. Radial growth rate (mm.day⁻¹) for each fungi matrix cultivated in MEA LP medium and Pagnocca B medium.

Fungi matrix	Culture medium	
	MEA LP	Pagnocca B
1	0.528	0.543
2	0.520	0.617
3	0.584	0.541
4	0.543	0.342
5	0.454	0.332
6	0.380	0.490
7	0.426	0.337
8	0.684	0.316
mean ± sd	0.515 ± 0.0957	0.440 ± 0.1208

These large differences among matrices could be explained taking into account that the mutualist is clonally propagated by queens that carry a pellet of the fungus in their mouth during their nuptial flight to establish new colonies (1). In addition to vertical propagation from one generation to

another, recent research has suggested that horizontal fungi transmission may also happen among ant nests or among close related ant species (12, 19).

Students’s T test was applied to verify whether there is a significant difference among daily fungal growth rates on petri dishes filled with MEA LP or Pagnocca B media. Since the observed t-value (T= 1.381) is below the absolute t-value (T = 1.7613), no significant difference has been found between daily growth rates using both MEA LP or Pagnocca B culture media.

The growth rate of *Leucoagaricus* species, mainly those with mutualistic relationship with leaf-cutting ants, has already been pointed out as very slow. The low growth rate has been considered a limiting factor regarding several experimental analyses (15; 21; 22). Loeck et al. (16) reported radial growth rate for the mutualistic fungus associated with another leaf-cutting ant species (*Atta sexdens piriventris*) with several culture media. The highest value obtained was 56.7 mm after 49 weeks or 0.165 mm.day⁻¹. This value is even lower than those obtained in the present work. Studies carried out with other non-mutualistic basidiomycetes species show values of growth rate at least 2,000 times higher in comparison with the mutualistic fungus used in this study. For example, Matheus (18) detected 0.90 ± 0.13 cm.day⁻¹ for *Agrocybe perfecta* (Rick) Sing., 1.14 ± 0.33 cm.day⁻¹ for *Coprinus jamaicensis* Murr., 2.53 ± 0.53 cm.day⁻¹ for *Pycnoporus sanguineus* (L:Fr.) Murr., and 3.77 ± 0.42 cm.day⁻¹ for *Phanerochaete chrysosporium* Burds. The intrinsic relationship inside higher attine nests could partially account for the low growth rate of the mutualistic fungus. The dynamics of the association between the ants and their fungi are complex, including other organisms such as the filamentous fungus *Pseudonocardia* (Actinomycetes) which produces antibiotics that inhibits the growth of *Escovopsis* (a virulent pathogen fungus), aiding in the garden maintenance (8).

Besides culture medium composition, other parameters have already been investigated concerning fungal growth rate improvement. Cazin *et al.* (5) tested three temperatures for *in vitro* culture, and observed higher growth rate at 24°C than at 30°C or 37°C. Our experiments have been conducted at 25°C ±

1°C, very close to the ideal temperature previously established.

Another problem regarding leaf-cutting ant mutualistic fungus culture is contaminants accumulation. This problem is closely correlated with the low growth rate of *Leucoagaricus* species in *in vitro* culture. Although reduced time for bioassays was the focus of some research (11, 21, 22), no ideal assay time can be defined based on the available data. Even though no contaminants have developed at most dish plates, in some bioassays lasting more than seven weeks changes were noted in the fungus color and in the culture medium, which were determinant for the experiment failure.

Distinct measurement ways have been used for determination of *in vitro* fungi growth. Although estimative evaluation based on visual observation (15, 22) and determination of relative percentage increase in comparison to the initial condition (9, 11, 21, 24) has been successful measurements, such criteria were dependent on the researcher interpretation. The measurement strategy used in the present work achieved more accurate results, since all values were obtained using a measure instrument. Loeck *et al.* (16) and Borba *et al.* (3, 4) have already employed the same radial-growth measures to evaluate the growth rate of the mutualist fungus of another leaf-cutting ant species.

Radial-growth rate has been shown to be a good measurement approach, although it does not take into account the fungal vertical growth or the increase of density in the Petri dish. Only radial growth (e.g. horizontal growth) is considered in this method. Notwithstanding the growth rate measurements presented have probably been underestimated, radial-growth use turns the obtained values easier to compare with other results and improve data accuracy.

Finally, since no significant difference was found between both tested culture media (MEA LP and Pagnocca B), none of the media can be considered more efficient than the other for fungal growth promotion. However, MEA LP is pointed out as more convenient, due its simpler composition and visual transparency, turning the radial measurements easier and more precise.

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Anexo 9

Myiashira, C.H., Tanigushi, D.G., Gugliotta, A., Santos, D.Y.A.C. Influence of caffeine on the survival of leaf-cutting ants *Atta sexdens rubropilosa* and *in vitro* growth of their mutualistic fungus. **Pest Management Science** 68: 935-940.

Influence of caffeine on the survival of leaf-cutting ants *Atta sexdens rubropilosa* and *in vitro* growth of their mutualistic fungus

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Abstract

BACKGROUND: Leaf-cutting ants collect plant fresh material for the cultivation of their mutualistic fungus. *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae) cause great economic losses through their foraging activity, mainly in agriculture. The main control method is the application of granulated toxic baits incorporated with an active ingredient (AI). The present goal is to evaluate the effect of caffeine on *in vitro* growth of the mutualistic fungus and on the survival of the leaf-cutting ants, aiming to verify the potential toxicity of this secondary metabolite over these organisms.

RESULTS: Three distinct patterns of fungal growth correlated with caffeine concentration were observed: (1) no effect (0.01% caffeine); (2) intermediate growth reduction (0.05% caffeine); (3) drastic growth reduction (0.10 and 0.50% caffeine). The highest caffeine concentration causes fungus death in the first week. As for insect survival, caffeine does not seem to exert any effect. The treatments with diet containing caffeine showed similar values of M50, irrespective of caffeine concentration.

CONCLUSION: As caffeine was shown to reduce growth of the mutualistic fungus of *Atta sexdens rubropilosa*, but with no conclusive effect on insect survival, a hypothetical explanation for the selection of different *Coffea* species by this leaf-cutting ant species might be associated with caffeine toxicity to the fungus.

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Keywords: leaf-cutting ants; mutualistic fungi; *Atta sexdens rubropilosa*; *Leucoagaricus gongylophorus*; caffeine

1 INTRODUCTION

Attine ants (subfamily Myrmicinae, tribe Attini) comprise a monophyletic group of more than 230 described species. They are found exclusively in the New World, with primarily neotropical distribution. All attine ants are obligatorily dependent on cultivation of fungus gardens for food.¹

Unlike more primitive attine ants, the leaf-cutting ants collect plant fresh material for the cultivation of the mutualistic fungus which has been identified as *Leucoagaricus gongylophorus* (Möller) Singer [= *Leucocoprinus gongylophorus* (Möller) Dörfelt & Creutzb.] by several authors.^{2–4} The insects feed on the gongylidia, special vesicle-like cells located at the end of hyphae, produced by the fungus. For larvae, the fungus is the sole food source, which provides them with a glycogen-rich diet for their growth and development. For adults of leaf-cutting ants, fungal ingestion represents less than 10% of their metabolic needs. Most nutrients are obtained through ingestion of plant sap.^{5,6}

Atta sexdens rubropilosa Forel (Hymenoptera: Formicidae), also known as lemon ants, cause great economic losses through their foraging activity, mainly in agriculture.⁷ Synthetic substances have been used to combat them. The main control method is the application of granulated toxic baits incorporated with an active ingredient (AI), which needs a delayed action to improve its effectiveness.⁸ However, these substances have significant

disadvantages, such as high cost and ecological impacts, which affect the entire biota.

Isolated compounds such as ricin,⁹ coumarins,¹⁰ lignans¹¹ or crude plant extracts^{12–14} have been studied as potential plant natural products for ant control, mostly looking for the effects of these compounds against the mutualistic fungus.

Caffeine (1,3,7-trimethylxanthine) is one of the most widely used plant secondary metabolites, primarily as a stimulant and an ingredient in drugs, found in more than 60 plant species. In nature, caffeine may function as an antiherbivory, and therefore it might be employed to protect agriculturally important crops. Recently, the simultaneous expression of three genes involved in caffeine biosynthesis in tobacco plants has yielded successful caffeine-producing transgenic plants unpalatable to tobacco cutworms (*Spodoptera litura* Fabricius).^{15,16}

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Larval development of *Aedes aegypti* L. (Diptera: Culicidae) has been interrupted when exposed to caffeine, with no evidence of developed resistance over insect generations undergoing the treatment. This compound has been employed as an alternative to the main control agents of this insect, as it has developed resistance to various drugs traditionally employed.¹⁷ The molluscicide effect of caffeine has also been pointed out.^{18,19}

Nevertheless, no positive correlation has been demonstrated between caffeine content and coffee genotype resistance against two important crop pests.^{20–22} Magalhães *et al.*²³ have even demonstrated that a higher caffeine content increases egg laying by the coffee leaf miner *Leucoptera* (= *Perileucoptera*) *coffeella* Guérin-Ménéville & Perrotet.

Caffeine antifungal activity has been demonstrated by several authors. Rizvi *et al.*²⁴ developed an *in vitro* experiment against the fungus *Helminthosporium maydis* Y. Nisik. & C. Miyake [= *Bipolaris maydis* (Y. Nisik & C. Miyake) Shoemaker], a pathogen of corn, and demonstrated the caffeine activity with a minimum inhibitory concentration of 1500 ppm. Arora and Ohla²⁵ reported that 0.5% caffeine solutions completely inhibited the growth of ten different species of wood-rotting fungi. Caffeine and caffeic acid exerted a negative effect on germination, on nuclear duplication cycle and on first septum formation on *Aspergillus nidulans* (Eidam) G. Winter.²⁶

The close relationship observed between attine ants and their mutualistic fungus has been the subject of several studies.^{27,28} The symbiotic relationship between these ants and the fungus they cultivate is obligate. As already stated, the fungus is the only source of food for ant larvae. Ants also benefit because fungus breaks down plant tissue, rendering nutrients available to them, and possibly detoxifies insecticidal plant compounds. The benefit to the fungus, in turn, is mainly related to its maintenance in an environment free of competition with other microorganisms.^{29,30} Thus, the fungus has some influence, although indirect, in the process of ant foraging.³¹ Species with fungicidal metabolites, such as terpenoids, are frequently rejected by leaf-cutting ants. Plant secondary metabolites may directly cause ant death by ingestion, or indirectly by reducing the size of their underground fungus gardens.

Based on field observations, it has been suggested³² that there is a correlation between susceptibility of coffee species to leaf-cutting ants and caffeine content. Mazzafera³² noted that susceptible ant-attack species of coffee have low caffeine concentrations in leaves. It has been observed (Affonso P, private communication, 2002) that rice flakes impregnated with high caffeine concentrations are rejected in pick-up tests, while lower concentrations of caffeine do not affect the foraging process.

In the present study, the effect of caffeine concentration on *in vitro* growth of the mutualistic fungus and on the survival of the leaf-cutting ants was evaluated with the aim of verifying the potential toxicity of this secondary metabolite on these organisms.

2 MATERIALS AND METHODS

2.1 Effect of caffeine on *in vitro* growth of the mutualistic fungus

The ant nests were maintained by the Phytochemistry Laboratory of the Institute of Bioscience (IB-USP), and the assays with the mutualistic fungus were performed at the Centre for Research in Micology of Botany Institute of São Paulo (IBt). Fifteen leaf-cutting ant nests maintained in the laboratory were used as the fungus source. The fungus culture was prepared as described in Miyashira

*et al.*³³ To evaluate the influence of caffeine, these fifteen matrices were split into two groups for two independent bioassays, A1 and A2, the former with eight matrices and the latter with seven matrices.

Each bioassay was developed with five treatments: the control situation (no caffeine) and four concentrations of caffeine (0.01, 0.05, 0.10 and 0.50%, based on the total weight of the culture medium). The culture medium (MEA LP) and the methodology used for measuring fungal growth were previously described.³³ The caffeine was dissolved in the same distilled water as that used in the culture medium.

The fungal growth data were obtained over an 8 week period. Data from the eighth week were used for radial growth rate (mm day^{-1}) and subjected to single-factor analysis of variance (ANOVA). With confirmation of the hypothesis that caffeine influenced fungus growth, data were subjected to Tukey's comparative test at 5% significance.

2.2 Effect of caffeine on ant survival

The method was similar to that described by Bueno *et al.*³⁴ and Howard *et al.*,³⁵ using solid diet. Caffeine was dissolved in distilled water and incorporated into diet at the same concentrations as those used for the fungus growth bioassay. The tests were conducted in petri dishes covered with filter paper moistened with 1 mL of distilled water and containing a small bottle with cotton moistened with 2 mL of water. Each petri dish received 400–500 mg of the solid diet per treatment. The filter paper, the bottle with cotton and the diet were replaced daily to avoid contamination by microorganisms. Seven treatments were tested: three controls (control 1: solid diet without caffeine + water; control 2: only water; control 3: no water or diet), and four with solid diets containing caffeine at 0.01, 0.05, 0.10 and 0.50% of total diet weight.

The bioassays to test ant survival were performed twice (B1 and B2). Each bioassay was done with six replicates (= ant nests, $n = 6$) for each treatment and ten ants per petri dish, a total of 60 ants for each treatment. Ants were collected from the nests and transferred to petri dishes, which were incubated at 24 °C and 70% relative humidity. The number of dead ants per petri dish was counted every 24 h.

The data of the day on which 50% of the ants were dead (M50) were subjected to ANOVA. When differences were detected, the data were subjected to Tukey's test at 5% significance.

3 RESULTS

3.1 Effect of caffeine on *in vitro* growth of the mutualistic fungus

Fungus growth in petri dishes was measured for 8 weeks. The final radial growth rates (RGRs) (Table 1) were subjected to ANOVA. Both bioassays, A1 ($F = 58.5267$, $F_{\text{critical}} = 2.6787$) and A2 ($F = 84.8465$, $F_{\text{critical}} = 2.6896$), showed that $F > F_{\text{critical}}$, revealing that caffeine concentration influences *in vitro* fungus growth. Data from each assay were subjected to Tukey's comparison test, which revealed that the data were statistically similar ($P > 0.05$) between control and 0.01% concentration, and between 0.10 and 0.50% concentrations. The RGRs in the caffeine concentration of 0.05% had values significantly different from any other concentration.

Based on these results, three distinct patterns of fungal growth correlating with caffeine concentration were observed. In the first pattern there is no effect of caffeine on fungus growth. The RGR

Table 1. Radial growth rate (RGR) (mm day^{-1}) of the mutualistic fungus of *Atta sexdens rubropilosa* nests cultivated in MEA LP medium incorporated with different dosages of caffeine

Treatment (% caffeine)	RGR (mm day^{-1}) (mean $\pm$ SD) ^a	
	A1	A2
Control	0.426 $\pm$ 0.190 a	0.493 $\pm$ 0.114 a
0.01	0.425 $\pm$ 0.081 a	0.483 $\pm$ 0.072 a
0.05	0.156 $\pm$ 0.116 b	0.224 $\pm$ 0.063 b
0.10	0.045 $\pm$ 0.055 c	0.044 $\pm$ 0.021 c
0.50	0.000 c	0.000 c

^a A1 and A2 correspond to the two bioassays. The same letters in the same column indicate no significative difference ($P \leq 0.05$).

of fungus on MEA LP with 0.01% caffeine was the same as that observed for the control (caffeine free). By contrast, higher caffeine concentrations (0.10 and 0.50%) caused a dramatic reduction in fungus growth. In the 0.50% caffeine assay, the initial inoculum died by the end of the first week. An intermediate concentration of caffeine (0.05%) set the third pattern, which presents a RGR with about 60% of growth reduction compared with the first group (Table 1, Fig. 1).

Besides the reduction in radial growth, there seems to be a delay in the fungal growth in treatments with 0.05 and 0.10% caffeine (Fig. 1). Radial fungus growth started later in these treatments when compared with the control and/or 0.01% caffeine conditions, with a delay ranging from 7 up to 21 days in the 0.10% treatment of bioassay A1.

3.2 Influence of caffeine on the survival of ants

In the same way as fungal growth analysis, two independent survival bioassays were performed with ants. In both bioassays, B1 and B2, analysis of variance showed that there were significant differences among M50 values (B1: $F = 12.597137$, $F_{\text{critical}} = 2.37178445$; B2: $F = 19.793351$, $F_{\text{critical}} = 2.37178445$), which means that treatments influence the survival of ants.

Data from each bioassay were subjected to Tukey's comparison test. The M50 in the treatment with solid diet and water (control 1) was the highest among all treatments for both bioassays (B1 and B2), 8.00 and 10.67 respectively, showing that leaf-cutting ants survived longer under such conditions. The M50 data for treatments with water and without diet (control 2: 4.67 and 4.33) and without water and diet (control 3: 4.83 and 4.50) were similar. The water availability does not seem to be a factor influencing the survival of ants, as the mortality in the control with water and without diet (control 2) and in the control without water and diet (control 3) was statistically similar for the two bioassays. Among

Figure 1. Radial growth rate (RGR) (mm day^{-1}) of the mutualistic fungus of *Atta sexdens rubropilosa* over time, cultivated at MEA LP medium incorporated with different dosages of caffeine. A: RGR for bioassay A1 ($n = 8$). B: RGR for bioassay A2 ($n = 7$). C: petri dishes with fungus culture after 8 weeks. Bar corresponds to 20 mm.

Table 2. Mean M50 (day in which 50% of the ants were dead) of *Atta sexdens rubropilosa* exposed to the survival bioassays (B1 and B2)

Treatment ^a	M50 (mean $\pm$ SD)	
	B1	B2
Control-1	8.00 $\pm$ 1.79 a	10.67 $\pm$ 1.51 c
Control-2	4.67 $\pm$ 0.52 b	4.33 $\pm$ 1.21 e
Control-3	4.83 $\pm$ 0.41 b	4.50 $\pm$ 0.55 de
0.01	4.17 $\pm$ 1.17 b	6.83 $\pm$ 2.64 d
0.05	4.83 $\pm$ 0.41 b	4.00 $\pm$ 0.63 e
0.10	4.67 $\pm$ 0.52 b	5.00 $\pm$ 0.63 de
0.50	5.00 $\pm$ 0.00 b	4.17 $\pm$ 0.75 e

^a Control 1: presence of water and solid caffeine-free diet; control 2: presence of water and absence of solid diet; control 3: absence of water and solid diet; 0.01: presence of water and solid diet with 0.01% caffeine; 0.05: presence of water and solid diet with 0.05% caffeine; 0.10: presence of water and solid diet with 0.10% caffeine; 0.50: presence of water and solid diet with 0.50% caffeine. The same letters in the same column indicate no significant difference ($P \leq 0.05$).

the treatments with diet containing caffeine, no wide variation in the M50 was observed, irrespective of the caffeine concentration (Table 2).

4 DISCUSSION

Fungus growth patterns in *in vitro* bioassays followed the same pattern as that observed by Affonso (private communication, 2002) after foraging pick-up tests. The author noted that flakes impregnated with the lowest caffeine concentration (0.01%) did not cause inhibition of ant foraging, while higher concentrations (0.015–0.7%) inhibited flake gathering. In fungus bioassays, a low caffeine concentration had no effect on fungus growth, while a high concentration completely inhibited fungus development.

Fungi are organisms that secrete digestive enzymes over food materials and feed on soluble products resulting from the extracellular digestion. The ability of caffeine degradation by some microorganisms, including fungi, by a demethylation route has already been emphasised by Dash and Gummadi.³⁶ The major byproduct formed by fungus caffeine degradation is theophylline, whereas theobromine is the major byproduct generated by bacterial metabolism. The delay observed in some treatments (Fig. 1) could be related to longer periods required for caffeine detoxification before the fungi start to absorb nutrients from the culture medium. Some authors have observed the occurrence of caffeine degradation by fungi using it as a nitrogen and/or carbon source.^{37–39}

Theobromine and theophylline are also plant secondary metabolites with some functions similar to those of caffeine. Nevertheless, no investigation has so far been made of the influence of such metabolites on the *in vitro* growth of the mutualistic fungus.

The inhibitory effect of caffeine was also assessed by Rizvi *et al.*²⁴ in an *in vitro* experiment with a parasitic fungus of maize (*Helminthosporium maydis*). The authors detected a minimum inhibitory concentration of 1500 ppm for that fungus and toxic effects on ten more fungus species at higher dosages. Fujii *et al.*⁴⁰ have shown the dose-dependent effect of caffeine on *Aspergillus flavus* Link, *A. niger* Tiegl., *A. ochraceus* G. Wilh., *Fusarium*

semitectum Berk. & Ravenel [= *Fusarium incarnatum* (Desm.) Sacc.] and *Penicillium* sp.

As caffeine was shown to reduce growth of the mutualistic fungus of *Atta sexdens rubropilosa*, the present authors speculate that the selection of different *Coffea* species by this leaf-cutting ant species, observed by Mazzafera,³² might be associated with caffeine toxicity to the fungus. However, the possibility of a direct toxic effect on the insect cannot be discarded.

In the B1 bioassay, the M50 observed in control 1, in which ants receiving solid diet without caffeine and water, proved to be the only ones among all treatments with a longer survival time. In the B2 bioassay, distinct values were detected between treatments. However, the data were not sufficient to suggest any kind of experimental influence on insects. Thus, the two bioassays can be considered to show similar results, as ants lived longer only when the caffeine-free diet was given. The mortality was higher or similar when the leaf-cutting ants were provided with diets containing any concentration of caffeine or in the absence of diet. Apparently, the availability of diet without potentially toxic/repellent compounds is crucial for longer survival of ants (Table 2).

Bueno *et al.*³⁴ subjected leaf-cutting ants to similar conditions. The M50 values obtained in the present bioassays for control 2 and control 3 are very close to those observed previously. Leaf-cutting ants subjected to control treatment without water and diet survived for 3 days according to Bueno *et al.*,³⁴ and for around 4 days in the present study (B1: 4.67 days; B2: 4.33 days) (Table 2). However, those authors were able to keep the ants alive longer (approximately 12 days) when subjected to control 1 treatment (solid diet caffeine free + water) as compared with the present bioassays (B1: 8.00 days; B2: 10.67 days). Although it is not possible to state whether or not the differences between these two studies are significant, both researches – the present work and Bueno *et al.*³⁴ – suggest that the ants supplied with diet present greater survival, while water supply does not influence this survival. Based on these observations, the small bottle with moistened cotton added to each petri dish could no longer be part of the experiment, making it simpler and less susceptible to microbial contamination.

The longer survival observed when solid diet was provided could be explained by the presence of glucose in the formulation of the diet. Glucose may be responsible for 50% of the nutritional needs of workers, as their main food source is plant sap.^{5,6} Silva *et al.*⁴¹ found higher survival rates of ants in experimental conditions, up to 25 days, when ants were subjected to high glucose diets.

The fragments of solid diet were changed daily. Careful observations revealed no evidence of cutting or biting on the surface of caffeine-containing diets, suggesting a repellence effect of this compound on ants. The use of caffeine in baits will require some attractive carrier (such as citrus juice) in an attempt to mask the presence of caffeine in diet.⁴²

The first direct evidence of the alkaloid caffeine as a chemical defence in plants was reported by Nathanson⁴³ using *Lycopersicon esculentum* (*Solanum lycopersicum* L.) $\times$ *Manduca sexta* L. as a model system.

Although leaf-cutting ants had been considered to be a problem in coffee plantations, Mazzafera³² pointed out that susceptible ant-attack species of coffee have low caffeine concentrations in leaves. Based on the present experimental model, it is not possible to establish whether caffeine has a toxic and/or repellent activity to ants. In contrast, the present results of *in vitro* bioassay with the mutualistic fungus show that 0.50% caffeine kills the fungus

inoculum just a few days after initial contact with the culture medium.

The data gathered here suggest that the selection of different *Coffea* species by leaf-cutting ants *Atta sexdens rubropilosa* is directed by the toxicity of caffeine to the mutualistic fungus, but the possibility of a direct toxic effect to the insect cannot be ruled out. The caffeine could be used as a fungicide in baits, mixed with some attractive carrier, and placed close to nest openings or along the trails.

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Anexo 10

Alonso, E.C., Santos, D.Y.A.C. 2013. *Ricinus communis* and *Jatropha curcas* (Euphorbiaceae) seed oil toxicity against *Atta sexdens rubropilosa* (Hymenoptera: Formicidae). **Journal of Economic Entomology** 106:742-746.

***Ricinus communis* and *Jatropha curcas* (Euphorbiaceae) Seed Oil Toxicity Against *Atta sexdens rubropilosa* (Hymenoptera: Formicidae)**

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Ricinus communis and *Jatropha curcas* (Euphorbiaceae) Seed Oil Toxicity Against *Atta sexdens rubropilosa* (Hymenoptera: Formicidae)

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ABSTRACT Leaf-cutting ants are the main herbivores in the New World tropics. Although the toxicity of seed oils against these ants has been poorly investigated, previous results revealed that seed oils exert considerable toxic activity against these insects. This paper analyzes the toxic action and deterrent properties of castor oil, *Ricinus communis* L., and physic nut oil, *Jatropha curcas* L., against workers of the leaf-cutting ant *Atta sexdens rubropilosa* reared in laboratory. Toxic effect was analyzed by feeding insects artificial diets supplemented with different oil concentrations and direct contact with the two oils. Deterrent activity was assessed by measuring the frequency of attendance to diets during the first 48 h of the ingestion bioassay. Castor oil at 10 and 30 mg/ml and physic nut oil at 5, 10, and 30 mg/ml were toxic by ingestion. In the direct contact bioassay, toxicity was observed for physic nut oil at 0.1 and 0.2 mg/ml, whereas castor oil exerted toxic effects only when the highest concentration was applied. Also, castor oil had a more pronounced deterrent effect against the leaf-cutting ant, compared with physic nut oil. Methods to apply these oils to control these insects are discussed.

KEY WORDS leaf-cutting ant, *Atta sexdens rubropilosa*, castor oil, physic nut, seed lipid

The leaf-cutting ants of the genus *Atta* are the main herbivores in the tropics of the New World and consume more plant material than mammals, caterpillars, or beetles (Wilson 1990). These ants rank among the most polyphagous and voracious herbivorous insects, cutting up to 15% of the leaves in the forest surrounding their colonies, every year (Urbas et al. 2007). In a ground-breaking study, Weber (1966) estimated a plant biomass of 5.9 tons as sustenance for a single colony of leaf-cutting ants over 77 mo. Ever since, the impact of this insect species' herbivorous habit has been the object of intense research (Rao et al. 2001; Corrêa et al. 2009). Generally, ants are beneficial to the environment, fertilizing soils, pollinating plants, and dispersing seeds (Bueno et al. 2005; Folgarait 1998). They also have been proven to play a role in nitrogen fixation (Pinto-Tomás et al. 2009). Nevertheless, in a scenario of increasing anthropic changes in the environment these insects are now considered pests, whose control is becoming more and more difficult (Bueno et al. 2005). In this sense, these ants pose a threat to silvicultural practices, plantations, pastures, and nurseries. Because of the competition with cattle, ants reduce the carrying capacity of rangeland and increase soil erosion (Vaccaro and Mousques 1997). Therefore, a useful control strategy would include the development of approaches to reduce populations down to levels that offset losses, without leading to eradication of the species (Guillade and Folgarait 2011). Several studies have been carried out using

plants in an attempt to obtain a natural product based control alternative to synthetic organic chemical insecticides, which are known to be harmful to the environment by decreasing populations of other insects including beneficials, and paving the way for the emergence of resistant pest strains (Peñaflor et al. 2009). However, an efficient large-scale control strategy has not been developed (Sumida et al. 2010).

The mass of vegetation removed by leaf-cutting ants is used inside the colony to grow a mutualistic fungus (Weber 1966), *Leucoagaricus gongylophorus* (= *Leucocoprinus gongylophorus*) (Fungi: Basidiomycota). The queen and larvae feed exclusively on the fungus, whereas workers rely almost entirely on the sap and nectar of the plants collected (Murakami and Higashi 1997; Mueller et al. 2005).

Numerous plants, including *Sesamum indicum* L. (Lamiales: Pedaliaceae), *Ipoema batatas* (L.) Lam. (Solanales: Convolvulaceae), and *Canavalia ensiformis* (L.) DC (Fabales: Fabaceae), have been tested for their harmful effects against leaf-cutting ants and their mutualistic fungus (Bueno et al. 1995; Hebling et al. 2000a,b). Bigi et al. (2004) reported toxic action of crude foliar extracts of *Ricinus communis* L. (Malpighiales: Euphorbiaceae) at 2 mg/ml by using injection test and comparing the 50% survival rate ($P < 0.001$). Lower concentrations of ricinine (0.2 and 0.4 mg/ml) caused similar effects.

Castor oil is extracted from the seeds of the castor bean plant and is used for therapeutic purposes in several countries, both topically and orally (Scarpa and Guerci 1980). Castor oil has considerable eco-

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Fig. 1. Leaf-cutting ant nests kept in the Laboratory of Phytochemistry, Department of Botany, Institute of Biosciences, USP. (A) Overview. (B) Fungus container. Photo: E.C.A.

conomic importance, and in Brazil it is also largely used in the production of biodiesel (Vaccaro et al. 2010). Apart from this, the oil is used as a raw material in different industries, and the pressed cake is used as fertilizer (Kouri et al. 2006). The main constituents of castor oil are ricinoleic (86%), linoleic (5.5%) and oleic acids (3.6%) (Martín et al. 2010). In turn, seeds of *Jatropha curcas* L. (Malpighiales: Euphorbiaceae) (physic nut) are used to extract an oil with significant potential applications in biodiesel production (Oliveira et al. 2009). In Latin America, physic nut oil is used mainly in cosmetic formulations, whereas the pressed cake is also used as fertilizer (GEXSI LLP 2008). This oil has been proven to be toxic to insects considered agricultural pests, as well as mollusks that act as disease vectors and fungi that cause mycoses (Gübitz et al. 1999; Adebawale and Adedire 2006). It is composed mainly of linoleic (39%), oleic (35.2%), palmitic (16.9%), and stearic (6.7%) acids (Martín et al. 2010). Seeds of the castor bean plant and of physic nut enjoy high yields in the extraction of constituent oils, ≈ 48 –55% by weight, respectively (Martínez-Herrera et al. 2005; Chakrabarti and Ahmad 2008). These percent yields are well above extraction figures observed for cotton (*Gossypium hirsutum* L.) or soybean [*Glycine max* (L.) Merr.], pointing to the higher economic potential of *R. communis* and *J. curcas* (Oliveira et al. 2009). Few studies have addressed the toxicity of seed oils and fractions thereof against leaf-cutting ants (Fernandes et al. 2002; Morini et al. 2005; Santos-Oliveira et al. 2006) and their mutualistic fungus (Fernandes et al. 2002). Nevertheless, these studies have shown that the seed oil extracted from these plants is significantly toxic to both organisms.

In this context, the current study assesses the toxic and deterrent effects of castor and physic nut oils against workers of the leaf-cutting ant *A. sexdens rubropilosa* reared in laboratory, with the goal being to answer two questions: 1) what is the mortality rate of

A. sexdens rubropilosa fed on castor and/or physic nut oils and 2) is *A. sexdens rubropilosa* deterred or attracted by castor and/or physic nut oils?

Materials and Methods

Biological Material. The ant nests used were maintained in the Laboratory of Phytochemistry, Department of Botany, Institute of Biosciences, University of São Paulo (USP). The nests are made of large plastic containers treated with talc (hydrous magnesium silicate, CAS no. 14-807-96-6) to prevent insects from escaping. Smaller pots were placed inside the large container, where ants cultivate fungus (Fig. 1). The room temperature ($24 \pm 1^\circ\text{C}$) was controlled with an air conditioner, and the humidity was kept between 70 and 80% with a humidifier. The ants were fed three times per week with leaves of copperleaf, *Acalypha wilkesiana* Mull.Arg. (Malpighiales: Euphorbiaceae), cultivated at the University garden, and maize, *Zea mays* L. (Poales: Poaceae) grits, obtained from a supermarket.

Approximately 50 g (fresh weight) of castor seeds ('IAC-Guarani', obtained from Instituto Agronômico de Campinas, SP, Brazil) were washed in dichloromethane and then crushed and submitted to exhaustive extraction in 500 ml of hexane by using a Soxhlet apparatus for 6 h, to obtain the respective oil. The extract was concentrated in a rotary evaporator under reduced pressure for total solvent removal. The same procedure was used to prepare the same amount of physic nut (RENASEM SP01487/2007, bought from Sementes Caiçara Ltda).

Bioassays. Sets of eight worker ants with cephalic capsule width between 2.0 and 2.8 mm were randomly selected from nests and transferred to petri dishes containing artificial diet, prepared with 5 g of glucose, 1 g of bacteriologic peptone, 0.1 g of yeast extract, and 1.5 g of agar in 100 ml of distilled water. The artificial

Fig. 2. Survival curves of workers of the leaf-cutting ant *Atta sexdens rubropilosa*. (A) Castor oil (*R. communis*) ingestion bioassay. (B) Physic nut oil (*J. curcas*) ingestion bioassay. (C) Castor oil contact bioassay. (D) Physic nut oil contact bioassay. Median survival time (S_{50}) is placed between brackets; different lowercase letters indicate significant differences between survival curves (data obtained using the log-rank test at $\alpha = 0.05$ and degrees of freedom = 3). Bars indicate SEs.

food source was provided inside a small aluminum foil cup. During the tests, the number of dead ants was recorded, and those individuals were removed (Bueno et al. 1997). The petri dishes were kept on a laboratory bench available in the same room as the nests. Bioassays were carried out with seven replicates (distinct nests).

Toxicity Bioassay by Ingestion. To measure the effect of the ingestion of oil by ants, three different concentrations of oil were added to artificial diet: 5, 10, and 30 mg/ml (Santos-Oliveira et al. 2006). Besides those treatments, a control group was fed oil-free artificial diet. Four petri dishes were prepared for each nest (replicate): 1) control, oil-free artificial diet; 2) treatment 1, artificial diet with 5 mg/ml oil; 3) treatment 2, artificial diet with 10 mg/ml oil; and 4) treatment 3, artificial diet with 30 mg/ml oil. All artificial diets, with the respective oil concentration, were replaced daily.

Preliminary Deterrence Bioassay. Ant activity of one replicate for each diet type was recorded during the first 48 h of the experiment for toxicity by ingestion. The aim was to assess putative deterrent activity of oils to ants, by monitoring the frequency of attendance of workers to the cups containing different diets (Santos-Oliveira et al. 2006).

Direct Contact Toxicity Bioassay. Topical toxicity of the castor and physic nut oils was measured using a micropipette to apply 1 μ l of three oil concentrations, 0.02, 0.1, and 0.2 mg/ml in hexane, according to Fernandes et al. (2002), to the pronota of ants (Santos-Oliveira et al. 2006). A solvent-control and a negative

control (no substance applied) were used. Oil-free diet was added in all petri dishes.

Data Analysis. Survival curves were analyzed using the log-rank test (Prism 5.04, GraphPad Software Inc., San Diego, CA). For each treatment, the median survival (S_{50}) was determined considering the day 50% worker survival was observed. The data obtained in the current study were compared with literature data considering the ratio between S_{50} of the treatment to S_{50} of the control.

Results and Discussion

Results of castor oil treatment revealed that only the 10 and 30 mg/ml concentrations were toxic to the ants, compared with the control ($P < 0.0001$). In turn, all treatments with physic nut oil were toxic, compared with the control ($P < 0.0001$ for treatments with 5 and 30 mg/ml and $P = 0.0110$ for treatment with 10 mg/ml) (Fig. 2A and B). As expected, in the current study the survival curves revealed higher mortality rates in ants treated with the highest oil concentrations (Fig. 2), except for treatment with physic nut oil 5 mg/ml, in which S_{50} concentration was lower than that obtained with 10 mg/ml. No conclusive explanation can be drawn from this finding. Although repetitions are needed, our preliminary data from the deterrence test show high attendance rates of ants to the 5 mg/ml physic nut oil diets (Fig. 3). The absence of any deterrence by physic nut oil, together with its toxic effect, could explain the lower S_{50} observed after exposure to 5 mg/ml oil, compared with exposure to 10

Fig. 3. Number of visits to the artificial diet in one of the seven replicates for each diet type in the oil ingestion bioassay.

mg/ml. According to this hypothesis, the insect would not be able to detect lower oil concentrations and would therefore forage the toxic diet indiscriminately, eventually dying. Granular baits are a common strategy used to control leaf-cutting ants in crop areas (Boaretto and Forti 1997). Considering the hypothesis above, physic nut oil could be a candidate as an active component to be used in the formulation of these baits. Another common strategy to control the leaf-cutting ant in agriculture is the application of insecticides directly in the nest (Zanetti et al. 2003). However, more in-depth field studies should be conducted to verify the efficacy of these seed oils as insecticides, considering different modes of application.

For >40 yr, the oil extracted from *Azadirachta indica* A. Juss (Sapindales: Meliaceae), commonly known as neem, has been used individually as a bioinsecticide and in the formulation of several products to control pests (Schmutterer 1990). In ingestion experiments similar to those conducted in the current study, Santos-Oliveira et al. (2006) observed that neem seed oil was toxic when applied as a 5 mg/ml solution ($S_{50} = 0.67$ of $S_{50 \text{ control}}$; $P < 0.05$), similarly to what we report herein for physic nut oil ($S_{50} = 0.50$ of $S_{50 \text{ control}}$; $P < 0.0001$) (Fig. 2B). However, direct contact with neem oil did not produce any toxic effect (Santos-Oliveira et al. 2006). In this aspect, both castor and physic nut oils were shown to have higher insecticidal activity. Physic nut oil at 0.1 mg/ml presented a $S_{50} = 0.57$ of $S_{50 \text{ solvent}}$ ($P = 0.0001$), and the same oil at 0.2 mg/ml led to a $S_{50} = 0.43$ of $S_{50 \text{ solvent}}$ ($P < 0.0001$), whereas castor oil at 0.2 mg/ml showed a $S_{50} = 0.18$ of $S_{50 \text{ solvent}}$ ($P < 0.0001$). All S_{50} values indicated that these concentrations were toxic to the ants (Fig. 2C and D). The solvent used to dilute the oil (hexane) was not toxic to insects, with toxicity very similar to that of the control.

Toxicity observed after topical treatment with castor and physic nut oils was similar to the values reported by Fernandes et al. (2002) for *Citrus* spp. (Rutaceae) seed oils, for which toxicity to leaf-cutting ants was attained at 0.2 mg/ml of oil ($S_{50} = 0.50$ of $S_{50 \text{ solvent}}$; $P < 0.01$).

Our results of deterrence assays show that attendance to the diet was inversely correlated with oil

concentration. This was more evident with castor oil, as compared with physic nut (Fig. 3). This result is similar to that reported by Santos-Oliveira et al. (2006), in a study that showed that the deterrent activity of neem oil was directly correlated to oil concentration in diets.

Arnosti et al. (2011) observed a toxic effect of ricinoleic acid, the main component of castor oil, preventing the development of oocytes of *Rhipicephalus sanguineus* (Latreille), suggesting an acaricidal potential. Rahuman et al. (2008) reported that oleic and linoleic acids, the main constituents of physic seed oil, are toxic to mosquito larvae. It is possible that these components are responsible for the toxicity of oils tested in the current study. In this sense, more detailed studies should be conducted to verify the toxicity of these components separately against the leaf-cutting ant *A. sexdens rubropilosa* and its mutualistic fungus, considering that combatting this organism could be achieved through a strategy based on the nutrition of the queen and her offspring.

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Anexo 11

Timich, M., Santos, D.Y.A.C. Effect of *Croton urucurana* Baill. extracts against *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae).
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Effect of *Croton urucurana* Baill. extracts against *Atta sexdens rubropilosa* Forel

(Hymenoptera: Formicidae)

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Running title: *Croton urucurana* extracts against leafcutter ants

ABSTRACT – (Effect of *Croton urucurana* Baill. extracts against *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae)) Leafcutter ants of the *Atta* genus cause serious problems in Brazilian agriculture. Currently, research efforts are directed to find a specific compounds to fight these ants, their symbiont fungus, or both. This study investigates whether direct topical application of hexanic, dichloromethanic, and methanolic leaf extracts of *Croton urucurana* affects mortality rate of *Atta sexdens rubropilosa* worker ants. In spite of previous research describing insecticide proprieties related to this *Croton* species, no significant difference was observed between mortality rates of ants treated with extracts and control. Although the *Croton* extracts were not toxic for ants, further bioassays with the symbiotic fungus should be conducted.

RESUMO – (Efeito de extratos de *Croton urucurana* Baill. sobre *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae)) As formigas cortadeiras do gênero *Atta* causam sérios problemas à agricultura brasileira. Atualmente, buscam-se substâncias que sejam específicas a essas formigas, ao seu fungo simbiote ou a ambos. O objetivo deste trabalho foi verificar se a aplicação tópica direta de extratos hexânico, diclorometânico e metanólico de folhas de *Croton urucurana* influenciam a taxa de mortalidade das operárias. Apesar de dados anteriores demonstrarem o efeito inseticida dessa espécie de *Croton*, não foi verificada diferença significativa na taxa de mortalidade das formigas tratadas com os extratos e o controle. No entanto, novos bioensaios usando o fungo simbiote devem ser realizados.

Keywords: *Croton urucurana*, Euphorbiaceae, leafcutter, bioassay, β -sitosterol

Introduction

Leafcutter ants of the *Atta* genus, *Attini* tribe, *Myrmicinae* family occur restrictedly in the Americas, distributed from central Argentina to southern USA (Herrera & Pellmyr 2002). These social insects live in nests built as a succession of chambers that are used to grow the symbiont fungus or to dispose of waste. These ants cause considerable damage to agriculture in Brazil (Fernandes *et al.* 2002) and together with ants of the *Acromyrmex* genus stand out as the main insect pest in tropical and subtropical regions in the American continent. It is estimated that between 12% and 175 of the total production of leaves in tropical forests is consumed by ants of the *Atta* genus, with a significantly higher impact on these environments, compared to any other herbivore (Herrera & Pellmyr 2002).

Currently, control strategies against this pest are based on unspecific manufactured insecticides that are harmful to non-target insects and contaminate the environment (Fernandes *et al.* 2002, Santos-Oliveira 2006). These side effects have prompted the search for insecticides with specific action against leafcutter ants, their symbiont fungus, or both (Fernandes *et al.* 2002).

Plants like *Spiranthera odorantissima* St. Hil (Terezan *et al.* 2010), *Azadirachta indica* A. Juss. (Oliveira *et al.* 2006) and *Citrus reticulate* Blanco (Fernandes *et al.* 2002) have been associated with proven toxic effect against *Atta sexdens rubropilosa* Forel.

Croton is a Euphorbiaceae genus that includes approximately 1,300 species distributed in tropical and subtropical regions of the New and the Old World (Govaerts *et al.* 2000). Plants of this genus have high levels of biologically active components, such as diterpenoids and alkaloids. Additionally, it comprises several aromatic species due to the presence of volatile oils (Salatino *et al.* 2007).

Croton urucurana Baill., also known as dragon blood, occurs in a wide area of the Brazilian territory, from the state of Bahia to Rio Grande do Sul and Mato Grosso (Salatino *et al.* 2007). Its popular name derives from the fact that the trunk releases red latex that is used as analgesic in folk medicine (Peres *et al.* 1997). Moreover, Silva *et al.* (2009) demonstrated that semipurified fractions of *C. urucurana* bark extracts significantly increased the mortality of *Anagasta kuehniella* Zeller (Lepidoptera: Pyralidae), suggesting the potential use as natural insecticide.

In this sense, the present study evaluated the effect of hexanic, dichloromethanic, and methanolic extracts of *C. urucurana* leaf extracts on the mortality of *Atta sexdens rubropilosa* workers using a direct contact bioassay.

Material and methods

Preparation of extracts

Ten grams of dry and finely powdered leaves of *C. urucurana* were submitted to 8-h serial extraction in a Soxhlet apparatus using hexane, dichloromethane, and methanol. Extracts were concentrated separately to dryness under reduced pressure in a rotatory evaporator and then diluted in the respective solvents to 0.5%, 1%, and 2% solutions.

Bioassays

The ant nests used are kept in the Laboratory of Phytochemistry, Department of Botany, Institute of Biosciences, University of São Paulo, into controlled room at $24 \pm 1^\circ\text{C}$ and 70% - 80% relative humidity. Ants are given a daily supply of leaves of *Acalypha wilkesiana* Mull Arg. and corn grits.

Five nests (replicates) were used during three bioassays, each one with five treatments: dry control (C1), solvent control (C2), treatment with 0.5% extract (T1), treatment with 1% extract (T2), and treatment with 2% extract (T3). For each replicate,

50 worker-ants (10 for each treatment) with cephalic capsule measuring between 2.0 mm and 2.5 mm were randomly collected and placed in a Petri dish with water and an artificial solid diet prepared with 0.1% yeast extract and 1.5% agar in 100 mL distilled water offered in a small plastic lid. The diet was replaced every 24 h to prevent contamination with microorganisms (Bueno *et al.* 1997).

Each ant was immobilized using tweezers. Then, 1 μ L of each extract at one of the concentrations used was dropped on the ant using a pipette (Fernandes *et al.* 2002). For the solvent control group, 1 μ L of pure solvent was used. Petri dishes were kept under the same conditions used for nests. The number of dead ants after exposure to treatments was recorded daily, for 25 days (Bueno *et al.* 1997).

Analysis of results

Survival rate of ants in each treatment was analyzed based on the day when 50% of ants were alive (S_{50}) (Alonso & Santos 2013). The data obtained with the five repeats of each treatment were analyzed using ANOVA to detect statistical differences. When these differences were observed, the *a posteriori* Tukey test was used to detect which treatment produced different results.

Analysis of secondary metabolites

The hexanic extract was submitted to gas chromatography–mass spectrometry (GC-MS) to identify the organic compounds in it. A HP-5MS column was used with injector temperature set at 250°C and the following heating gradient: 4 min at 150°C, a 150°C increase to 320°C at 6°C/min, and 2 min at 320°C. The substances were identified comparing mass and relative intensity of peaks with data available in the MassBank.jp online databank (<http://massbank.jp>).

Results and Discussion

Comparing both dry (C1) and solvent (C2) controls, no statistically significant

differences were observed with all three solvents used (Table 1). The absence of considerable deleterious effects by applying solvents topically onto leafcutter ants has already been reported in other studies. Similar findings were observed by Fernandes *et al.* (2002) in a study that evaluated the effect of the oil of citric fruit seeds diluted in hexane and ethyl acetate, and by Alonso & Santos (2013), in an investigation of the efficiency of hexanic extracts from seeds of two Euphorbiaceae species.

Here, as a rule, the leaf extracts of *C. urucurana* did not exhibit insecticide effect against *Atta sexdens rubropilosa* worker ants (Table 1). The S_{50} values observed for all treatments did not differ from dry or solvent controls. At dry control, the S_{50} values ranged from 7.5 to 10 days depending on the bioassay. Bueno *et al.* (1997) have already found S_{50} of 10 days in a similar assay. Despite the absence of statistical difference, a slight decrease at S_{50} values was noted with higher doses of hexanic and dichloromethanic extracts, but not for the methanolic extract (Table 1).

In spite of the higher mortality in *Anagasta kuehniella* larvae observed by Silva *et al.* (2009) with the exposure to *C. urucurana* extracts, the insecticidal action of this plant could not be confirmed in *Atta sexdens rubropilosa*.

Although no statistical significance was observed among the S_{50} for all bioassays, the data obtained for the exposure to hexanic extracts in the present study pointed out an interesting pattern of survival. Looking through survival curves, there is a visual distinction of dry-control ants (C1) comparing to the others (C2, T1, T2, T3) (Fig. 1). The number of survivors was always higher from day 1 until day 21. Comparing the survival on a daily basis, a significant difference was observed in S_{50} of ants from the dry control and the other treatments between days 3 and 6 (day 3 $P > 0.0504$; day 4 $P > 0.027$; day 5 $P > 0.0142$ and day 6 $P > 0.0327$), reinforcing that visual difference. For dichloromethanic and methanol extracts no distinction were found throughout the

bioassays.

Dutra *et al.* (2011) reported on the cytotoxic activity of hexanic and dichloromethanic extracts of *C. urucurana* against *Artemia salina*. Moreover, these extracts also exhibited *in vitro* antibacterial action (Oliveira *et al.* 2008). The presence of β -sitosterol-O-glycoside and of other substances in the methanolic, hexanic, and hydroalcoholic extracts of *C. urucurana* bark was pointed out to be behind the bactericidal action against *Staphylococcus aureus* (Peres *et al.* 1997). Among other minor compounds, the GC-MS revealed β -sitosterol as an important constituent of hexanic extracts of *C. urucurana*.

Finally, before abandoning the use of extracts of *Croton urucurana* as an alternative control of leafcutter ants, further bioassays investigating the toxic effect of these extracts could be performed. Miyashira *et al.* (2012) using distinct doses of caffeine detected high toxic effect of this compound against the symbiotic fungus but none effect against the insect. Since there is a strict dependency between this insect and the symbiotic fungus, the use of a fungicide incorporated into baits could be an interesting alternative to actual unspecific insecticides.

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Table 1. Mean day when 50% (S_{50}) of leafcutter ants survived, with different bioassays.

C1: dry control; C2: control with solvent; T1: extract 0.5%; T2: extract 1%; T3: extract 2%. Identical letters in the same column indicate the absence of significant differences.

S_{50} hexane = $p > 0.4307$, S_{50} dichloromethane = $p > 0.5663$, S_{50} methanol = $p > 0.8893$.

Treatment	S_{50} hexane	S_{50} dichloromethane	S_{50} methanol
C1	10 $\pm$ 1.936 a	7.5 $\pm$ 1.095 b	8 $\pm$ 2.987 c
C2	8 $\pm$ 1.850 a	9 $\pm$ 2.274 b	10 $\pm$ 35.579 c
T1	8.5 $\pm$ 2.244 a	9 $\pm$ 3.489 b	10 $\pm$ 3.475 c
T2	7.5 $\pm$ 2.387 a	8.5 $\pm$ 3.817 b	11 $\pm$ 2.151 c
T3	6.5 $\pm$ 3.061 a	7.5 $\pm$ 2.043 b	9 $\pm$ 3.347 c

Figure legend

Fig 1. Survival curve of *Atta sexdens rubropilosa* workers submitted to the bioassays of topical toxicity with extracts of *Croton urucurana* leaves. A. Assay with hexanic extract. B. Assay with dichloromethanic extract. C. Assay with methanolic extract.

Fig 1. Curva de sobrevivência de operárias de *Atta sexdens rubropilosa* submetidas aos bioensaios de toxicidade tópica com extratos foliares de *Croton urucurana*. A. Ensaio com extrato hexânico. B. Ensaio com extrato diclorometânico. C. Ensaio com extrato metanólico.

Fig. 1