UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Papel do alcalóide braquicerina na resposta ao estresse por radiação ultravioleta e dano mecânico em *Psychotria brachyceras* Müll Arg

Diogo Denardi Porto

Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS como requisito parcial para a obtenção do grau de Doutor em Ciências.

Orientador:

Prof. Dr. Arthur Germano Fett-Neto

Porto Alegre

fevereiro de 2009

Instituições e Fontes Financiadoras

O presente trabalho foi realizado no Laboratório de Fisiologia Vegetal, pertencente ao Departamento de Botânica do Instituto de Biociências da Universidade Federal do Rio Grande do Sul (UFRGS) e ao Centro de Biotecnologia da UFRGS.

Os auxílios financeiros foram obtidos do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), e da Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

Banca examinadora

Dr. Paulo Mazzafera

Instituto de Biologia, Departamento de Fisiologia Vegetal
Universidade Estadual de Campinas

Dr. José Cláudio Fonseca Moreira

Instituto de Ciências Básicas da Saúde, Departamento de Bioquímica
Universidade Federal do Rio Grande do Sul

Dr. Giancarlo Pasquali

Programa de Pós-Graduação em Biologia Celular e Molecular
Centro de Biotecnologia
Universidade Federal do Rio Grande do Sul

Dra. Janette Palma Fett

Membro suplente da banca examinadora
Programa de Pós-Graduação em Biologia Celular e Molecular
Centro de Biotecnologia
Universidade Federal do Rio Grande do Sul

À Fabiana e à Ana Clara

"Em algum lugar, algo incrível aguarda
ser descoberto."
Carl Sagan

Agradecimentos

Uma infinidade de pessoas contribuiu em maior ou menor grau para a realização deste trabalho. Muitas delas, algumas até sem perceber, me revelaram um caminho através da névoa que se forma no meio de tanta informação.

Agradeço, para começar, ao meu orientador Arthur. Foram-se nove anos. Entre Iniciação Científica e Pós-Graduação, nove anos de *Psychotria brachyceras*. E no entanto, esse projeto fantástico me colocou em contato com praticamente tudo que a biotecnologia tem a oferecer, e ainda com alguns aspectos de ciências farmacêuticas. O professor Arthur é uma fonte de valores da boa Ciência, de quem aprendi como é ser um profissional e um cientista responsável.

Aos meus colegas de Laboratório de Fisiologia Vegetal, esse pessoal que torna a rotina mais leve e ao mesmo tempo contribui para um trabalho de mais peso. Às "psicótricas" Paloma e Naíla, muita indiada nós três fizemos, nas coletas, congressos e etc. Aos amigos e suas fanfarronices Raul, Ricardo, Felipe, Vinícius e Guilherme, grandes companhias, desde que não se saia do recinto esquecendo o e-mail aberto... Aos meus orientados de Iniciação Científica Denílson, Laura, Camilo e em especial ao Hélio que, "na moral", tem todos os ingredientes para sair do forno como um profissional de sucesso.

Ao pessoal da Central Analítica e do Laboratório de Farmacognosia da Faculdade de Farmácia, técnica científica Ana Aboy, Dra. Miriam A. Apel e Dra. Amélia Henriques, sem as quais a análise de braquicerina não seria possível. Também na Faculdade de Farmácia, meus calorosos agradecimentos à professora Grace Gosmann e sua equipe do Laboratório de Fitoquímica, Simone Quintana, Juliane, Fernanda e Anna, as três últimas também colegas de Fisiologia Vegetal, muito obrigado por serem pacientes e atenciosas.

À minha Comissão de Acompanhamento, Dr. Carlos Termignioni e Dra. Célia Carlini. Apesar de estarem adicionalmente ocupados à época do meu doutorado

(diretor do Centro de Biotecnologia e Coordenadora do PPGBCM, respectivamente), atenderam prontamente às minhas dúvidas e necessidades.

Aos secretários do Programa Sílvia e Luciano, por sua calma organização e incansável simpatia.

À segurança da UFRGS pelas escoltas em coletas no Morro Santana.

À banca examinadora desta tese, Dr. Paulo Mazzafera, Dr. José Cláudio F. Moreira e Dr. Giancarlo Pasquali.

À Dra. Lúcia R. B. Vargas (UCS), que generosamente nos forneceu indivíduos de *Spodoptera frugiperda* para uso nos experimentos.

À professora Dra. Janette Palma Fett, pela revisão da tese e pelos conselhos para a bancada e para a paternidade.

Ao pessoal do Laboratório de Proteínas Tóxicas, pelo uso do insetário e por auxílios em geral.

Ao Luiz e à Janine, sô, compadre e comadre, amigos, confidentes, conselheiros, enfim, valiosos amigos.

Falando em amigos, ao Rodrigo Lorenzi, Rafael Lopo, Eduardo "Labareda", Fabio Hecht, Rodrigo Chaves, Éderson "Iguana", Ricardo Duarte e Daniel Christian. Eterna amizade ao som de Kinks, Beatles e Doors.

À minha mãe, por ser minha orientadora em quesitos emocionais, afetivos, financeiros, nutricionais, psicossociológicos... a lista é grande. Com ela aprendi sobre perseverança, disciplina e responsabilidade.

À Fabiana, pela confiança, pelo carinho, pela paciência, pela convivência, e à Ana Clara, pela existência. Vocês duas me fazem sentir único, importante, capaz, e fico feliz e orgulhoso por dividir minha vida com vocês.

ÍNDICE

LISTA DE ABREVIATURAS

AMIs Alcalóides Monoterpeno-Indólicos

CLAE Cromatografia Líquida de Alta Eficiência

DAB 3,3-diaminobenzidina

DW Dry weight

HPLC High-performance Liquid Chromatography

KJ Kilojoule

NBT Nitroblue Tetrazolium

SPSS Statistical Package for Social Sciences

TDC Triptofano descarboxilase

UV ultravioleta (comprimentos de onda de 180 a 400 nm)

UV-A ultravioleta-A (comprimentos de onda de 320 a 400 nm)

UV-B ultravioleta-B (comprimentos de onda de 280 a 320 nm)

UV-C ultravioleta-C (comprimentos de onda de 180 a 280 nm)

v/v relação volume/volume

RESUMO

As plantas superiores sintetizam uma ampla variedade de compostos, denominados classicamente metabólitos secundários, que as ajudam a se adaptar ao seu ambiente. Um subconjunto de metabólitos secundários são os alcalóides. Essas substâncias nitrogenadas de baixo peso molecular desempenham funções variadas nas plantas, podendo apresentar propriedades antibióticas, repelentes de herbívoros ou alelopáticas.

O alcalóide monoterpeno indólico braquicerina, de estrutura inédita, foi extraído da planta arbustiva *Psychotria brachyceras* (Rubiaceae). Esse composto revelou ação específica como antiinflamatório em testes de quimiotaxia, possuindo, portanto, potencial valor farmacológico. Trabalhos anteriores revelaram que a molécula possui significativas propriedades antioxidantes e antimutagênicas, e que a concentração foliar desse alcalóide é induzida por dano mecânico e radiação ultravioleta (UV).

Neste trabalho, demonstramos os resultados de uma série de experimentos desenvolvidos com o objetivo de entender o papel de braquicerina em *P. brachyceras* frente a esses estresses. Uma parte dos experimentos foi desenvolvida paralelamente com amostras de *Psychotria carthagenensis* da mesma região, que não acumula alcalóides e é mais sensível a UV do que *P. brachyceras,* além de ser alvo significativo de herbívoros em seu ambiente natural.

O comportamento de algumas classes de metabólitos secundários reconhecidas como importantes na resposta a ferimento e radiação UV é descrito em amostras de *P. brachyceras* submetidas a esses tratamentos. A aplicação de dano mecânico e de radiação ultravioleta não alterou os níveis de compostos fenólicos e flavonóides totais, respectivamente. A única classe de metabólitos que sofreu regulação nessas condições foram antocianinas, que tiveram sua concentração induzida em experimentos de exposição à UV; porém, os teores induzidos em *P. brachyceras* foram muito menores do que àqueles basais em *P.*

carthagenensis. Os dados sugerem um papel predominante do alcalóide frente a esses estresses.

O potencial tóxico/repelente da braquicerina foi testado em bioensaios que utilizaram herbívoros generalistas. O alcalóide isolado não foi capaz de inibir o consumo das amostras pelos animais, o mesmo ocorrendo com extratos de *P. brachyceras.* Porém, extratos de *P. carthagenensis* inibiram a alimentação do molusco *Helix aspersa*, provavelmente pela presença de taninos. A atividade protetora do alcalóide foi testada em discos foliares de *P. carthagenensis* expostos à UV. A aplicação de braquicerina diminuiu a degradação de clorofilas causada pela radiação. Ensaios de atividade antioxidante *in vitro* e *in vivo* demostraram que o alcalóide é ativo em relação ao ânion superóxido e ao peróxido de hidrogênio, espécies ativas de oxigênio importantes no estresse por ultravioleta e dano mecânico, respectivamente. Além disso, foi verificado que epidermes foliares acumulam braquicerina.

Os dados sugerem uma atividade predominantemente antioxidante ou moduladora de estresse oxidativo para o alcalóide. Essa atividade de detoxificação pode fazer parte de uma estratégia de tolerância da planta tanto em relação ao ultravioleta quanto ao ferimento/herbivoria e pode ser importante em outras condições adversas.

ABSTRACT

Higher plants synthesize a wide range of compounds, classically known as secondary metabolites, which help plants adapt to their environment. A class of secondary metabolites are the alkaloids. These nitrogen-containing low molecular weight substances play several roles in plants as antimicrobial, herbivore deterrents or allelopathic agents.

The monoterpene indole alkaloid brachycerine, which has a unique structure, was extracted from the shrub *Psychotria brachyceras* (Rubiaceae). This compound showed antinflamatory activity in chemotaxis assays, indicative of pharmacological potential. Previous work described antioxidant and antimutagenic effects for brachycerine, and the regulation of its leaf tissue concentration upon ultraviolet (UV) radiation exposure and mechanical damage stress.

In this thesis, we report the data from a series of experiments in an attempt to understand the roles of brachycerine in *P. brachyceras* with regard to wounding and UV stresses. Some experiments were carried on using samples from *Psychotria carthagenensis* shrubs of the same region, a plant devoid of detectable alkaloids. *P. carthagenensis* leaves are UV-sensitive if compared to *P. brachyceras* and are often found damaged by herbivores in field-grown plants.

The contents of secondary metabolites with known functions in wounding and UV responses are described from experiments in which *P. brachyceras* samples were submitted to these treatments. The only group of secondary metabolites regulated by these stresses was that of anthocyanins in UV treated samples; however, induced content of these metabolites were always much lower than the basal ones in *P. carthagenensis*. The data indicated a major role for brachycerine in UV and wound responses.

Toxic/deterrent properties of brachycerine were tested in bioassays with generalist herbivores. The alkaloid failed to deter herbivore feeding, however, samples treated with *P. carthagenensis* extracts were less consumed by the snail *Helix aspersa,* and this may be due to the presence of tannins. UV-protecting

activity was tested in brachycerine-treated *P. carthagenensis* leaf disks exposed to UV radiation. Brachycerine treatment protected disk samples from UV-driven chlorophyll loss. *In vitro* and *in vivo* assays detected antioxidant activity of brachycerine towards superoxide anion and hydrogen peroxide, reactive oxygen species related to UV and wounding stress, respectively. In addition, it was shown that leaf epidermal layers accumulate brachycerin.

The results suggest an antioxidant or oxidative stress modulator role for brachycerine in *P. brachyceras.* This detoxifying activity may be part of a tolerance strategy in relation to UV and wounding stresses, and perhaps to other environmental pressures.

INTRODUÇÃO

1. METABÓLITOS SECUNDÁRIOS

A condição séssil, característica das plantas, demanda mecanismos eficientes de resposta a sinais e estresses ambientais. Em comparação com espécies dotadas de mobilidade, plantas são mais afetados por variações e limitações impostas pelo ambiente e respondem às mesmas com diversas estratégias de ordem fisiológica (Huey *et al.,* 2002).

Um conjunto de substâncias produzidas pelas plantas e de grande importância nesse aspecto são os metabólitos secundários. Também referidos como produtos naturais, esses metabólitos compreendem um amplo leque de substâncias das mais variadas estruturas e de diferentes origens metabólicas. O isolamento da morfina em 1806 é considerado o início dos estudos com metabólitos secundários de plantas, e esses compostos foram, a princípio, considerados subprodutos indesejados do metabolismo (Hartmann, 2007). Atualmente, mais de 200,000 estruturas de metabólitos secundários já foram desvendadas, e sabe-se que esses compostos têm a função de incrementar o desempenho das plantas frente às condições ambientais a que estão submetidas (Hartmann, 2007; Bourgaud *et al.,* 2001). De forma simplificada, os metabólitos secundários vegetais podem ser divididos em três grandes grupos: os compostos fenólicos, os terpenóides e os compostos secundários nitrogenados.

1.1 Compostos fenólicos

Os compostos fenólicos são encontrados em todas as espécies de plantas ditas "superiores", ou seja, gimnospermas e angiospermas. Essas espécies produzem lignina, polímero importante na sustentação da estrutura da planta e formado por monômeros de compostos fenólicos. A presença de lignina nos tecidos vegetais foi essencial no processo de colonização da terra por plantas ancestrais (Burgoyne *et al.,* 2005). Além da sustentação, os fenólicos exercem muitas outras funções nas plantas, inclusive relacionadas à defesa (Dixon & Paiva, 1995). Os compostos fenólicos, presentes em frutas, hortaliças e certas bebidas, são o grupo mais estudado de metabólitos secundários e têm grande importância na prevenção de várias doenças humanas devido à sua

atividade antioxidante (Boudet, 2007). A biossíntese da maioria dos compostos fenólicos depende da atividade da fenilalanina amônia liase. Essa enzima catalisa a remoção do grupo amônia desse aminoácido, inutilizando-o para a síntese de proteínas e direcionando-o para a defesa e sinalização em tecidos vegetais. Por ser um ponto de divisão entre o metabolismo primário e secundário e desviar aminoácidos da síntese regular de proteínas para defesa celular, essa etapa é altamente regulada (Boudet, 2007).

Uma importante classe de compostos fenólicos são os flavonóides. São compostos distribuídos universalmente em angiospermas, gimnospermas e pteridófitas, presentes nos órgãos das partes aéreas, ou seja, folhas, flores e frutos (Harborne, 1993; Cushnie & Lamb, 2005). O esqueleto básico dos flavonóides está ilustrado na Figura 1.

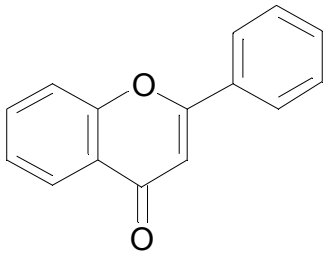


Figura 1: Esqueleto molecular dos flavonóides.

Essas substâncias têm diversas funções nas plantas (Harborne & Williams, 2000). Em flores, a presença dos flavonóides confere coloração às pétalas, em faixas de cor que variam de branco ao violeta e aproximam-se do preto. A cor dependerá principalmente do flavonóide específico sendo acumulado e de sua concentração. A coloração das pétalas das flores de uma planta tem influência decisiva em sua estratégia de polinização, visto que as diferentes espécies animais polinizadoras são atraídas por cores distintas (Harborne, 1993).

Outra função bem estabelecida dos flavonóides é sua ação antimicrobiana (Harborne & Williams, 2000). Em plantas, é comum a indução do acúmulo foliar de flavonóides em resposta ao ataque de patógenos, e essa função encontra confirmação em outros testes, como, por exemplo, na inibição da germinação de esporos fúngicos (Cushnie & Lamb, 2005).

As populações humanas também se beneficiam das propriedades antimicrobianas dos flavonóides. Preparações produzidas a partir de plantas ricas em flavonóides usadas para fins medicinais, como própolis, são usadas há séculos, com registros datando da época de Hipócrates (460-377 a. C., Cushnie & Lamb, 2005). Vários flavonóides são ativos até mesmo contra o vírus causador do HIV humano (Lameira *et al.,* 2006). Atualmente, a busca por novos medicamentos de combate a infecções é de extrema necessidade, e os flavonóides podem contribuir com novas estruturas para essa finalidade.

1.2 Terpenóides

Os terpenóides são compostos secundários formados a partir da condensação de unidades de cinco carbonos, o isopentenil pirofosfato e o dimetilalil fosfato (IPP e DMAPP, respectivamente; Figura 2). O IPP e o DMAPP são derivados de pelo menos duas rotas de síntese; a rota citoplasmática do mevalonato, que deriva da condensação de 3 moléculas de acetil-CoA, e a rota plastídica do metil-eritrol-fosfato, iniciada pela condensação de uma molécula de piruvato com uma de glicerol-3-fosfato (Taiz & Zeiger, 1999).

Figura 2: Isopentenil-pirofosfato (esquerda) e dimetilalil-pirofosfato (direita), precursores dos terpenóides.

Os terpenóides são amplamente distribuídos nos vegetais, sendo constituintes importantes de óleos essenciais, látex e resinas, e desempenham importante papel na defesa vegetal contra herbívoros devido a suas propriedades tóxicas e repelentes (Harborne, 1993), além de várias outras funções. São a maior e mais diversa classe de metabólitos secundários, com estimativas de mais de 55 mil estruturas elucidadas atualmente (Maimone & Baran, 2007; Carvalho & Fonseca, 2005).

Um dos critérios de classificação dos terpenóides é o número de carbonos utilizados na síntese de sua estrutura (Bakkali *et al.,* 2008). Dessa forma, temos os hemiterpenos (5 carbonos), monoterpenos (10), sesquiterpenos (15), diterpenos (20),

triterpenos (30), tetraterpenos (40 carbonos) e politerpenos (acima de 40 carbonos). Alguns mono e sesquiterpenos são voláteis e podem participar da defesa por um mecanismo indireto, atraindo predadores e parasitas do herbívoro pelo qual a planta está sendo atacada (Turlings *et al.,* 1995). Além de participantes da defesa vegetal, os mono e alguns sesquiterpenos são importantes componentes nos compostos voláteis de flores responsáveis pela atração de polinizadores (van Schie *et al.,* 2006).

Pesquisas preliminares apontavam uma hipotética divisão do fornecimento de precursores entre as duas rotas de síntese de IPP para as diferentes subclasses de terpenóides. A rota citosólica resultaria nos sesqui e triterpenos, enquanto a rota plastídica seria responsável pelos mono, di e tetraterpenos. Essa divisão foi confirmada através de ensaios com precursores marcados radioativamente (Dudareva *et al.,* 2004), embora não necessariamente da forma como hipoteticamente postulada, pois existe um certo grau de sobreposição entre estas vias metabólicas, dependendo do órgão e status fisiológico da planta. Em flores de *Antirrhinum majus*, a rota plastídica é a responsável pelo fornecimento de IPP e DMAPP para o conjunto de terpenos voláteis da planta, que inclui alguns monoterpenos e um sesquiterpeno (Dudareva *et al.,* 2004).

Os terpenos são usados em diversas aplicações, desde cosméticos até a indústria da aviação. Em vista disso, um grande interesse é direcionado para a síntese química de terpenos, e muitas moléculas já são totalmente sintetizadas em laboratório (Maimone & Baran, 2007). Outra fonte alternativa de terpenos é a biotransformação, que consiste na utilização de sistemas vivos para a obtenção de novas estruturas (Carvalho & Fonseca, 2005). Esse método é interessante porque as reações ocorrem sob condições brandas, ou seja, temperatura e pressão próximas às ambientais, tornando o processo relativamente barato.

1.3 Compostos nitrogenados

As plantas apresentam uma grande variedade de compostos secundários nitrogenados, e a principal função deles é a defesa contra os herbívoros (Harborne, 1993). Os de estrutura mais simples são os aminoácidos não-protéicos, análogos de aminoácidos comuns. Essas moléculas são tóxicas porque são incluídas na síntese protéica do herbívoro, gerando proteínas não-funcionais. Os aminoácidos não-protéicos são

freqüentemente acumulados em sementes de legumes, podendo ser usados pela planta como fonte de nitrogênio, constituindo assim uma exceção quanto às funções principais de metabólitos secundários.

Outras classes de compostos secundários nitrogenados acumulados por certas plantas são os glicosídeos cianogênicos e os glicosinolatos. Quando os tecidos de plantas são atacados por herbívoros, os glicosídeos cianogênicos liberam ácido cianídrico, molécula altamente tóxica que interfere no transporte de elétrons da respiração celular, além de conferir sabor amargo. Como o ácido cianídrico é tóxico também para as plantas, os glicosídeos são quebrados enzimaticamente apenas em caso de destruição das membranas (Harborne, 1993). Os glicosinolatos, muito comuns em plantas da família Brassicaceae, são também inativos na forma intacta, acumulando-se preponderantemente nos vacúolos. Com a ocorrência de dano mecânico, enzimas do tipo mirosinases são ativadas e atacam os glicosinolatos, geralmente produzindo isotiocianatos, os quais conferem sabor pungente, inibindo herbivoria (Taiz e Zeiger, 1999).

Os alcalóides são os compostos nitrogenados mais conhecidos na função de defesa contra herbívoros (Harborne, 1993). São definidos como pequenas moléculas que possuem um anel heterocíclico com nitrogênio (Taiz & Zeiger, 1999), uma definição ampla que comporta uma grande variedade de estruturas. Os alcalóides estão presentes em cerca de 20% das espécies vasculares, e são conhecidos e usados por humanos desde a antiguidade. O filósofo grego Sócrates foi envenenado com coniina, um alcalóide extremamente tóxico encontrado em extratos de *Conium maculatum*. A atropina, também encontrada nessa planta, mas principalmente em *Atropa belladonna*, era usada por mulheres medievais com finalidade cosmética, pois causa a dilatação das pupilas. Atualmente, a atropina tem diversos usos na medicina e é citada pela Organização Mundial da Saúde como um dos 15 medicamentos essenciais (WHO, 2007). Outros exemplos de alcalóides de uso difundido são a nicotina, a cocaína e a morfina.

Os alcalóides são derivados principalmente da descarboxilação de aminoácidos ou intermediários da síntese de aminoácidos (Taiz & Zeiger, 1999, De Luca & Laflamme, 2001) pela atividade de enzimas como a triptofano descarboxilase e a tirosina descarboxilase (Facchini *et al.,* 2000). Essas enzimas modificam os aminoácidos de forma a transformá-los em precursores para a produção dos alcalóides, desviando-os do processo de síntese de proteínas. Essa importante influência da produção de alcalóides

nos processos metabólicos, direcionando reservas de nitrogênio - um importante e muitas vezes escasso nutriente em condições naturais - e interferindo na síntese protéica, torna compreensível a necessidade de regulação exercida sobre o acúmulo dessas moléculas. Diversos estudos indicam que a produção de alcalóides é altamente regulada. Os alcalóides de vinca (*Catharanthus roseus*), por exemplo, derivam de uma rota biossintética cujas enzimas estão localizadas separadamente nas raízes, epiderme e idioblastos de folhas, flores e caules (De Luca & St. Pierre, 2000). Em papoula (*Papaver somniferum*), planta produtora de alcalóides importantes como morfina e codeína, a primeira enzima de síntese de alcalóides, tirosina descarboxilase, encontra-se expressa principalmente em raízes, enquanto que as enzimas subsequentes têm maior presença no caule (Facchini & Park, 2003).

Além de distribuídas diferencialmente entre órgãos, as enzimas de biossíntese de alcalóides podem ter separação espacial entre as organelas celulares. A biossíntese de alcalóides de várias classes depende de enzimas cuja localização varia entre o citosol, o cloroplasto e o retículo endoplasmático (Facchini & St Pierre, 2005). Uma provável função para essa separação é o controle dos efeitos tóxicos dos intermediários da biossíntese dos alcalóides (Pasquali *et al.,* 2006). A triptamina, por exemplo, causa lesões necróticas quando seu acúmulo é induzido pela expressão ectópica de triptofano descarboxilase em folhas de tabaco (Di Fiore *et al.,* 2002).

2. ALCALÓIDES MONOTERPENO-INDÓLICOS

São vários os aminoácidos que podem ser modificados de forma a dar origem a alcalóides em plantas. Os alcalóides, por sua vez, são classificados de acordo com o aminoácido que lhes deu origem (De Luca & Laflamme, 2001). Os alcalóides derivados do aminoácido triptofano são chamados de alcalóides indólicos, devido ao anel presente na cadeia lateral do triptofano, chamado de indol. O passo enzimático inicial da biossíntese dos alcalóides indólicos está ilustrado na Figura 3.

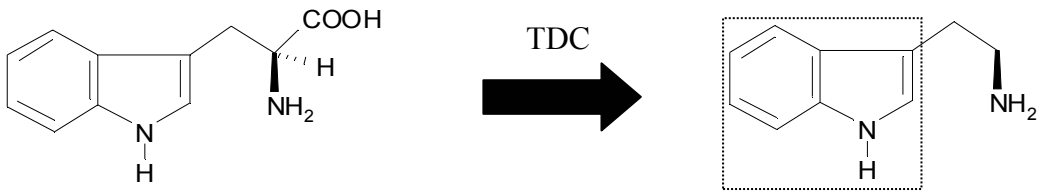


Figura 3 - A descarboxilação do aminoácido triptofano (esquerda) pela enzima triptofano descarboxilase (TDC) produzindo o proto-alcalóide triptamina (direita) é o início da biossíntese dos alcalóides indólicos. O anel indólico está marcado com o quadrado pontilhado.

A descarboxilação do triptofano não tem como única função a síntese de alcalóides. O anel indólico pode ser utilizado para a síntese das principais auxinas das plantas e muitos outros compostos (Cano *et al.,* 2003).

Freqüentemente os alcalóides indólicos apresentam origem mista. Uma importante classe de alcalóides indólicos é formada a partir da condensação do precursor indólico (triptamina) e de um precursor da rota dos terpenos. A essa classe dá-se o nome de alcalóides monoterpeno-indólicos (AMIs). Os AMIs são encontrados apenas em plantas das famílias Apocynaceae, Loganiaceae, Rubiaceae e Nyssaceae (Memelink *et al.,* 2001) e incluem compostos de grande importância farmacêutica. Alguns AMIs de destaque são a vincristina e a vinblastina, agentes quimioterápicos amplamente utilizados no tratamento de alguns tipos de câncer. Esses dois alcalóides são produzidos pela vinca, *Catharanthus roseus.*

2.1 Os alcalóides de Catharanthus roseus

Devido à sua grande importância na medicina, os AMIs de *C. roseus* são amplamente estudados (Pasquali *et al.,* 2006). A planta tem potencial genético para sintetizar mais de 100 estruturas alcaloídicas (Memelink *et al.,* 2001). Apesar de a estrutura molecular e a rota de síntese desses alcalóides já estar elucidada e grande parte dos genes codificadores das enzimas dessa rota terem sido clonados, a principal fonte comercial de vincristina e vinblastina é o plantio extensivo de *C. roseus* e a purificação dos alcalóides a partir de tecidos vegetais adultos. A necessidade atual desse processo vem da grande complexidade química desses alcalóides, tanto da vincristina e vinblastina per se quanto de sua rota de biossíntese, que envolve diversos passos enzimáticos, o que dificulta sobremaneira a síntese química (Fig. 4).

Outra característica da biossíntese dos AMIs de *C. roseus* que dificulta sua reprodução em sistema controlado é a distribuição dos passos da rota por diferentes ambientes celulares (Murata *et al.,* 2008). As primeiras etapas de síntese, passando pela condensação que forma a strictosidina até a 16-hidroxi-tabersonina, ocorrem na epiderme. Em seguida, esse intermediário é transportado pelas células do parênquima paliçádico e chega a idioblastos e laticíferos, onde as formas finais, vincristina e vinblastina, são sintetizadas e armazenadas.

3. OS METABÓLITOS SECUNDÁRIOS NA MEDICINA

As plantas medicinais têm sido usadas como remédios por milhares de anos, e essa prática persiste em populações tradicionais, sendo também comum em países como a China e a Índia. O isolamento de compostos ativos a partir de tecidos vegetais surgiu no início do século XIX (Balunas & Kinghorn, 2005). Com a revolução industrial e o desenvolvimento da química orgânica, a indústria de medicamentos passou a priorizar os compostos de origem sintética, por serem de fácil obtenção e purificação. Ainda assim, atualmente cerca de um quarto dos medicamentos prescritos mundialmente são de origem vegetal (Rates, 2001; Balunas & Kinghorn, 2005).

Existem aproximadamente 250 mil espécies de plantas, entretanto apenas cerca de 10% dessas espécies passaram por algum teste de atividade biológica, e uma fração ainda menor foi submetida a análises químicas mais detalhadas (Harvey, 2000). Essa lentidão pode ser derivada tanto da complexidade química dos extratos vegetais quanto da dificuldade de acesso à biodiversidade, concentrada em países tropicais menos desenvolvidos e freqüentemente sem legislação específica nesse sentido. A estratégia inicial de busca por compostos naturais de potencial farmacêutico (bioprospecção) mais usada é a etnofarmacológica e etnobotânica (Rates, 2001; Balunas & Kinghorn, 2005). O processo consiste na identificação e estudo de espécies vegetais utilizadas por populações tradicionais, com o objetivo de realizar posteriormente o isolamento de princípios ativos a partir dessas plantas.

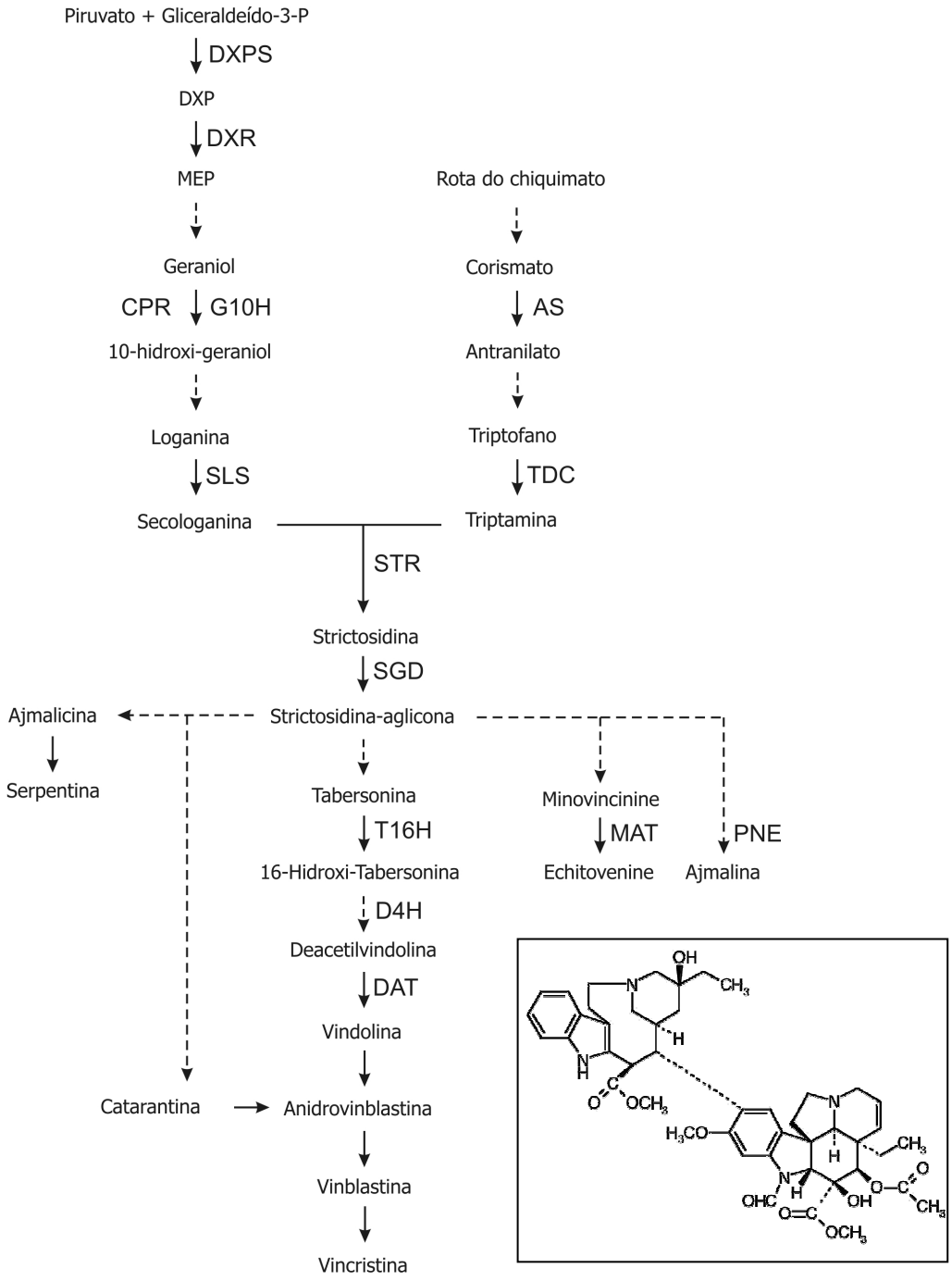


Figura 4 - Rota de biossíntese dos alcalóides de *Catharanthus roseus.* Setas de linha contínua representam um passo enzimático, setas de linha pontilhada indicam dois passos ou mais. DXPS: desoxi-xilulose fosfato sintase, DXP: desoxi-xilulose fosfato, DXR: desoxi-xilulose fosfato reductoisomerase, MEP: metil-eritrol fosfato, CPR: citocromo 450-redutase, G10H: geraniol 10-hidroxilase, AS: antranilato sintase, SLS: secologanina sintase, TDC: triptofano descarboxilase, STR: strictosidina sintase, SGD: strictosidina glucosidase, T16H: tabersonina 16-hidroxilase, MAT: minovincinine-19-hidroxi-*o*-acetiltransferase, PNE: polineuridina aldeído esterase, D4H: desacetoxivindolina 4-hidroxilase, DAT: desacetilvindolina acetiltransferase. Na caixa, a estrutura molecular da vincristina. Adaptado de Pasqualí *et al.,* 2006.

O uso terapêutico dos alcalóides de *C. roseus* é um exemplo emblemático da bioprospecção farmacêutica. A espécie é nativa e endêmica da ilha de Madagascar, e populações indígenas utilizam a planta para tratamento de enfermidades como diabetes e febre (Gurib-Fakim, 2006). Estudos químicos detalhados da planta revelaram seu amplo leque de AMIs e a potente atividade anticâncer de alguns deles, com índices de regressão tumoral de 80% para a doença de Hodgkin tratada com vinblastina e de 90% para leucemia tratada com vincristina (Balunas & Kinghorn, 2005). Entretanto, as dificuldades encontradas na produção dos alcalóides de *C. roseus*, juntamente com o grande interesse comercial associado à sua eficiente ação anticâncer, levou a uma exploração predatória da planta. Estima-se que a população natural de *C. roseus* tenha sido quase completamente exterminada devido à grande demanda pelos medicamentos. Atualmente a espécie é plantada comercialmente. Portanto, é crucial que a extensão do valor da biodiversidade, tanto como potencial genético quanto por sua influência no equilíbrio ambiental, sejam conhecidos tendo em vista o melhor controle de sua exploração (Koo & Wright, 1999).

Além da possibilidade de valorização do conhecimento de populações indígenas ou aborígenes, que serve como uma espécie de teste clínico preliminar, o fato de a biodiversidade ser um ótimo ponto de partida para a prospecção farmacêutica tem a ver com a natureza das moléculas produzidas por organismos vivos. A maioria desses metabólitos passou por um longo processo de evolução que resultou no aperfeiçoamento de sua afinidade por alvos de ação biológicos, como proteínas, carboidratos e ácidos nucléicos. Além disso, seu tamanho molecular reduzido muitas vezes facilita a permeabilidade através de membranas, e sua síntese baseada em maquinarias protéicas auxilia sua metabolização por processos enzimáticos (Wilson & Danishefsky, 2006). Outra propriedade interessante dessas moléculas é o fato de sua função e estabilidade terem sido adaptadas, durante o processo evolutivo, a ambientes celulares. Essa propriedade é importante nos estudos farmacocinéticos de novos agentes terapêuticos.

4. O POTENCIAL FARMACÊUTICO DAS ESPÉCIES DE *Psychotria* DO SUL DO BRASIL

Utilizando-se da estratégica etnobotânica de bioprospecção, espécies utilizadas para fins medicinais por caboclos da Amazônia foram investigadas pelo grupo da

professora Elaine Elisabetsky e colaboradores (Elisabetsky *et al.,* 1995). Várias espécies de *Psychotria* sp. foram documentadas como ativas no tratamento de enfermidades, de erupções cutâneas a dores diversas. Em especial, preparações baseadas em flores de *Psychotria colorata* foram relatadas como eficientes no tratamento de dores abdominais e otites. Dados iniciais confirmaram a ação analgésica e apontaram para compostos alcaloídicos como responsáveis por essa atividade (Elisabetsky *et al.,* 1995). Caracterizações químicas detalhadas revelaram que os tecidos de *P. colorata*, em especial as flores, acumulam pelo menos sete alcalóides diferentes (Verotta *et al.,* 1998) e três deles apresentaram atividade analgésica específica (Verotta *et al.,* 1999).

O gênero *Psychotria* é extremamente amplo. De origem austro-asiática, compreende 1000 a 1650 espécies e caracteriza-se pelo acúmulo de alcalóides indólicos (Nepokroeff *et al.,* 1999). Os dados de *P. colorata* motivaram uma busca por compostos de potencial farmacêutico entre os alcalóides de *Psychotria* sp. do sul do Brasil (Verotta *et al.,* 1999; Both *et al.,* 2002b). O Rio Grande do Sul abriga 18 espécies nativas de *Psychotria*, e a investigação química de algumas dessas plantas revelou diversas estruturas inéditas. Entre as espécies produtoras de alcalóides, aparecem *P. suterella* (Santos *et al.,* 2001), *P. brachyceras* (Kerber *et al.,* 2001), *P. leiocarpa* (Henriques *et al.,* 2004), *P. myriantha* (Simões-Pires *et al.,* 2006) e *P. umbellata* (Kerber *et al.,* 2008). Das seis espécies investigadas, apenas uma não apresentou alcalóides, *P. carthagenensis* (Leal & Elisabetsky, 1996). Curiosamente, partes aéreas *P. carthagenensis* são utilizadas no sul do Brasil em substituição à *P. viridis* no preparo do "santo daime", uma bebida alucinógena. A princípio, alcalóides indólicos seriam o princípio ativo alucinogênico, portanto a ausência deles nos tecidos de *P. carthagenensis* parece sugerir um outro mecanismo psicotrópico (Leal & Elisabetsky, 1996). Porém, a ausência de alcalóides nessa planta na região não implica necessariamente em sua ausência em outros locais de ocorrência.

Alguns alcalóides e/ou extratos alcaloídicos dessas espécies mostram atividades farmacológicas promissoras. A psicolatina de *P. umbellata*, antes conhecida como umbelatina, apresentou atividades analgésica (Both *et al.,* 2002a) e psicoativa (Both *et al.,* 2005) específicas em testes com camundongos. Além dessas, nosso grupo demonstrou propriedades antioxidantes e antimutagênicas do alcalóide (Fragoso *et al.,* 2008). Extratos de *P. brachyceras, P. leiocarpa, P. suterella* e *P. myriantha* demonstraram atividade

analgésica não-dose dependente (Both *et al.,* 2002b). O extrato de *P. myriantha* apresentou ainda atividade antiinflamatória em testes com camundongos (Simões-Pires *et al.,* 2006).

O potencial genético das espécies de *Psychotria* não está restrito apenas à produção de alcalóides. Culturas in vitro de *P. brachyceras* são capazes de converter alfa-pineno, um terpeno obtido em grande quantidade a partir da resina de *Pinus* sp., em verbenol e verbenona, terpenos com várias aplicações comerciais e, portanto, com alto valor agregado (Limberger *et al.,* 2007).

5. BRAQUICERINA, UM ALCALÓIDE MONOTERPENO INDÓLICO BIOATIVO DE *Psychotria brachyceras*

A espécie *Psychotria brachyceras* Mull. Arg. possui hábito arbustivo e distribuição bastante ampla. Sua maior ocorrência é em zonas próximas ao litoral, especialmente entre os municípios de Osório e Torres - RS, em Mata de Planície Litorânea, onde atinge até 3 metros de altura e tem floração mais exuberante do que em outros locais (Dillenburg & Porto, 1985). A partir de partes aéreas da planta foi isolado o alcalóide monoterpeno indólico braquicerina (Fig. 5a, Kerber *et al.,* 2001). Testes de migração de leucócitos em ratos revelaram a braquicerina como um antiinflamatório (Henriques, A. T., dados não publicados).

A rota de biossíntese proposta para a molécula de braquicerina assemelha-se àquela descrita para a strictosidina de *Catharanthus roseus* (Fig. 5b, Gregianini *et al.,* 2004). A molécula pode estar sendo formada na planta através da condensação da triptamina com um iridóide da rota dos terpenos, possivelmente 1-epiloganina (Kerber *et al.,* 2001). No entanto, essa hipótese tem sido criticada (Søren R. Jensen, Technical University of Denmark, comunicação pessoal) com a alternativa de o iridóide da porção terpênica ser a 10-hidroxi-loganina (Kamiya *et al.,* 2002). De qualquer forma, a estrutura do alcalóide apresenta peculiaridades, como a origem a partir de terpeno diferente de secologanina e manutenção de um resíduo de glicose em sua forma final. Estas características são incomuns para alcalóides indólicos, e isso abre caminho para novos estudos de rotas biossintéticas nessa classe de substâncias, bem como novos potenciais farmacológicos.

Até o momento, sabemos que o conteúdo foliar de braquicerina é sensível a alguns fatores de estresse. A concentração do alcalóide nas folhas de estacas de *P. brachyceras* aumenta com a incidência de luz ultravioleta (UV) de curto comprimento de onda (254 nm), podendo ultrapassar oito vezes o conteúdo basal encontrado em folhas crescidas a campo (Gregianini *et al.,* 2003); indução mais modesta foi observada com UV-B (280 a 320 nm). Na busca de uma explicação para esse fato, observamos que a braquicerina, além de absorver fortemente na faixa do UV, tem atividade antioxidante de oxigênio singleto *in vitro* (Gregianini *et al.,* 2003).

O dano mecânico é outro fator capaz de elicitar alterações do conteúdo do alcalóide em folhas. Estacas submetidas a esse tratamento dobram sua concentração foliar de braquicerina no segundo dia após o dano. Buscando rotas de sinalização que possam estar atuando nessa resposta, descobrimos que o metil-jasmonato, um hormônio vegetal envolvido principalmente em respostas à herbivoria (Wasternack, 2007), é capaz de induzir a produção de braquicerina em folhas de estacas de *P. brachyceras* (Gregianini *et. al.,* 2004).

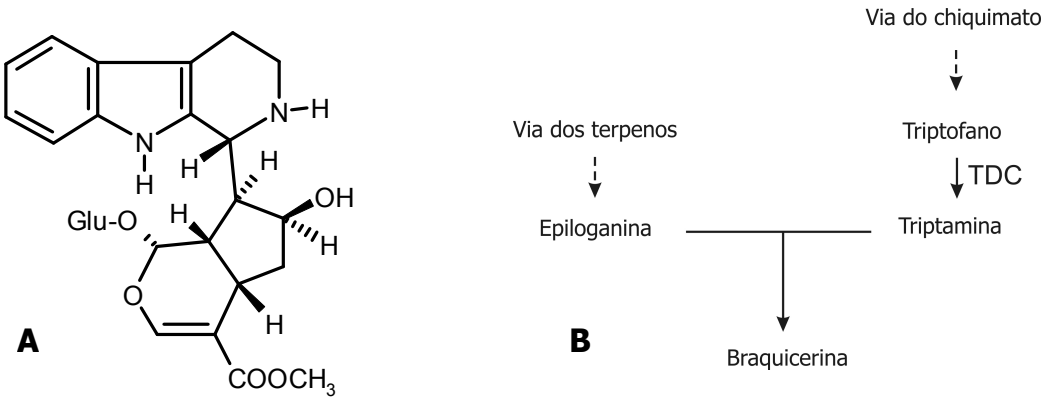


Figura 5 – A: Estrutura do alcalóide monoterpeno-indólico braquicerina. **B:** Rota hipotética de biossíntese de braquicerina em *Psychotria brachyceras.* TDC: triptofano descarboxilase.

6. OBJETIVOS

Tendo em vista a base teórica estabelecida sobre a função de alcalóides em plantas e os dados obtidos sobre a regulação da produção de braquicerina em *Psychotria*

brachyceras, bem como a potencial aplicação de braquicerina como agente terapêutico, este trabalho teve como objetivos gerais:

- Analisar a cinética das respostas espaço - temporais de biossíntese do alcalóide braquicerina em relação a estímulos de UV e ferimento em *Psychotria brachyceras*;
- Comparar o perfil de acúmulo de braquicerina com o de outros metabólitos secundários comumente relacionados a respostas a UV e herbivoria em outras espécies vegetais, investigando se há alguma coordenação da indução das vias de braquicerina e de outros metabólitos;
- Caracterizar o papel de braquicerina no contexto das principais adaptações bioquímicas e estruturais de proteção contra excesso de UV-B e como repelente de herbívoros generalistas em *Psychotria brachyceras*.

FORMATO E ORGANIZAÇÃO DA TESE

A presente tese de Doutorado está dividida em duas linhas de investigação: a linha relacionada a dano mecânico e herbivoria, e a linha voltada às respostas frente à radiação ultravioleta. Ambas as linhas deram origem a dados que serão apresentados no formato de manuscritos que serão enviados a periódicos especializados. Na **Parte I,** são descritos os dados referentes aos experimentos relacionados ao dano mecânico e herbivoria, e a **Parte II** contém os resultados decorrentes de experimentos com radiação ultravioleta. No final da tese consta uma discussão geral, resultados principais e a bibliografia completa.

PARTE I : DANO MECÂNICO E HERBIVORIA

MANUSCRITO 1 : The wounding-induced antioxidant indole alkaloid brachycerine of *Psychotria brachyceras* is not a deterrent, but plays an indirect chemical defense role in response to damage

Artigo a ser submetido ao periódico Journal of Chemical Ecology

The wounding-induced antioxidant indole alkaloid brachycerine of *Psychotria brachyceras* is not a deterrent, but plays an indirect chemical defense role in response to damage

Diogo Denardi Porto[1], Hélio Nitta Matsuura[2], Amélia Terezinha Henriques[3], Arthur Germano Fett-Neto[1,2] *

[1]Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, CP 15005, Porto Alegre, Rio Grande do Sul, 91501-610, Brazil.

[2]Departamento de Botânica, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

[3]Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

Herbivory tolerance mediated by brachycerine in *Psychotria brachyceras*

E-mail address: fettneto@cbiot.ufrgs.br (A. G. Fett-Neto), Phone 55-51-3308-7642, Fax: 55-51-3308-7309

ABSTRACT

Plants are often the targets of herbivore attack, and accumulation of secondary metabolites may deter the associated tissue losses. Alkaloids and tannins are well characterized as toxic compounds in many plant-herbivore interactions. Wounding or jasmonate application promote leaf accumulation of brachycerine, a major shoot-specific monoterpene-indole alkaloid produced by *Psychotria brachyceras*. The highest concentrations of the alkaloid are found in inflorescences. Brachycerine has antimutagenic properties in yeast, as well as strong *in vitro* antioxidant capacity, being capable of quenching singlet oxygen, hydroxyl radical, and superoxide. This study aimed at characterizing the putative role of brachycerine in *P. brachyceras* responses to wounding and herbivory. Damage applied to leaves increased the content of brachycerine locally, without causing significant changes in the alkaloid level in stems. Wounding did not affect the content of total phenolics in *P. brachyceras*, and the species yielded negative results for tannin detection. These data suggest an *in situ*, non-systemic role for the damage-inducible alkaloid in plant defense. However, generalist herbivore bioassays (with *Spodoptera frugiperda* and *Helix aspersa*) failed to show toxic effects of brachycerine or *P. brachyceras* extracts. On the other hand, an *in vivo* hydrogen peroxide staining assay, with both leaf disks and detached leaves of *Coleus blumei*, showed that alkaloid treated tissues accumulated less wound-generated peroxide. Taken together, data indicate that brachycerine is not a herbivore deterrent, but rather an indirect chemical defense, modulating oxidative stress derived from mechanical damage.

Keywords: Psychotria brachyceras, Psychotria carthagenensis, wounding, herbivory, secondary metabolites, alkaloid, brachycerine, peroxide.

INTRODUCTION

Plant responses to the attacks of herbivores may involve different strategies that differ according to the species examined. The herbivores, on their turn, must overcome the plant defenses in order to use their tissues for feeding. Elrich and Raven (1964) proposed the concept of co-evolution and the understanding of plant-insect interactions by an arms race metaphor. Many studies from then on followed this thought framework and provided explanations for a multitude of ecochemical phenomena (Després et al., 2007).

Plant defenses can be direct and indirect (Kessler and Baldwin, 2002). Indirect defenses involve attraction of herbivore predators, which may be mediated by volatile organic compounds released by plants under attack (Turlings et al., 1995; Arimura et al., 2000). Direct defenses are traits that affect herbivore feeding behavior and/or performance, e.g. thorns, accumulation of protease inhibitors or secondary metabolites (Kessler and Baldwin, 2002).

Alkaloids are small nitrogen-containing molecules, often derived from amino acids (De Luca and Laflamme, 2001). Production and storage of these substances by plants is well documented as a defense response to herbivore and pathogen attacks (Siciliano et al., 2005; Wittstock and Gershenzon, 2002; Gatehouse, 2002). Phenolic compounds, another class of secondary metabolites, are a large group of substances with diverse functions in plants (Boudet, 2007). Phenolics can accumulate in response to various abiotic and biotic stresses, including wounding and herbivory (Dixon and Paiva, 1995). Tannins are an important group of phenolic compounds. These substances are characterized by the capacity of binding proteins and causing precipitation. Tannins are often effective in the

protection of plant tissues from insect (Xiao et al., 2007) and mammal herbivores (Makkar, 2003).

Another strategy developed by plants to cope with herbivory stress is tolerance (Kessler and Baldwin, 2002; Stowe et al., 2000). Herbivory tolerant plants sustain tissue loss by diminishing its effects with mechanisms like resource relocation, increased photosynthetic and growth rates and increased branching (Strauss and Agrawal, 1999). Mechanical wounding associated with folivory elicits the production of H_2O_2 (Orozco-Cardenas and Ryan, 1999), which, if not controled, may cause disruption of cell structures. Accumulation of antioxidant compounds such as anthocyanins in leaves may alleviate the wound-generated oxidative stress (Gould et al., 2002) and contribute to plant tolerance to herbivory.

Psychotria brachyceras Mull. Arg. (Rubiaceae) is a southern Brazilian native shrub. This plant accumulates a shoot-specific monoterpene indole alkaloid called brachycerine (Kerber et al., 2001). This alkaloid shows antioxidant and antimutagenic properties, accumulates to high concentration in inflorescences, and its concentration in leaves is inducible by ultraviolet radiation, wounding and jasmonate treatments (Gregianini et al., 2003; Gregianini et al., 2004; Nascimento et al., 2007). Induction by wounding and jasmonate, an important signal molecule triggering defenses against herbivore attack (Wasternack, 2007), suggested the involvement of this alkaloid in herbivory responses. In order to test if brachycerine had a role in defense against herbivory, a series of experiments was carried out to analyse the responses and effects of brachycerine with respect to wounding and herbivory. For comparison purposes, some experiments were also performed using samples of *P. carthagenensis*, a co-occurring closely related species which does not produce alkaloids (Leal and Elisabetsky, 1996). Incorporation of brachycerine in

heterologous systems, such as variegated *Coleus blumei* leaves, was also done to examine *in situ* peroxide production in wound zones both in presence and absence of the alkaloid.

MATERIALS AND METHODS

Plant material and wounding treatments. Tip stem cuttings from adult trees of *P. brachyceras* and *P. carthagenensis* grown in the understorey of a subtropical forest located at Morro Santana – UFRGS (Porto Alegre, Rio Grande do Sul, Brazil) were used in the experiments. The cuttings were washed and acclimatized in hydroponic medium composed of MS salts (Murashige and Skoog, 1962) at 10% strength for 10 days before treatments.

After acclimatization, stem cutting leaves (3 out of 4) were mechanically wounded four times each with tweezers (two wounds per side of the leaf blade). Damage to mid vein and larger secondary veins was avoided. Leaves were left intact in control cuttings. Samples (full set of leaves from each cutting) were collected immediately and at 2, 4, 8, 12, 24, 48 and 72 h after damage, frozen in liquid nitrogen and stored at -20°C until analysis.

Brachycerine isolation. Authentic brachycerine was isolated according to Kerber et al. (2001) with a few modifications. *P. brachyceras* leaves were collected from adult plants grown in the field and air dried under shade at room temperature (25-30°C) for one week. Leaf material (ca. 400 g) was extracted in commercial ethanol (5 l) with a blender and macerated in the dark for 3 weeks. The suspension was occasionally shaken and the ethanol was changed weekly. The ethanolic extract was then filtered through qualitative filter paper and evaporated to dryness in a rotary evaporator (Buchi Laboratory Equipments, Flawil, Switzerland). The dried extract was ressuspended in 0.1 M HCl and washed with methylene

chloride in separatory funnel. The pH of the aqueous phase was then raised to 10.0 with ammonium hydroxide and total alkaloids were extracted in methylene chloride with a soxhlet. After concentration, total alkaloids were separated by liquid chromatography in a flash column using as solvent chloroform:methanol (95:5) with increasing methanol concentrations. The fraction 70:30 yielded authentic brachycerine as confirmed by analysis with RP-HPLC (LC-20A, Shimadzu Corporation, Kyoto, Japan), previously reported (Gregianini et al., 2003).

Total soluble phenolics estimation. Soluble phenolics were extracted and assayed as described (Fett-Neto et al., 1992). Leaf tissue samples (50 mg) were ground with liquid nitrogen and total soluble phenolics were extracted for 30 min in a sonic bath in 1.5 ml ice-cold 0.1 M HCl. The samples were then centrifuged at 10 000 g for 30 min at 4°C. The supernatant was recovered and the sediment was re-extracted. The combined extracts were made up to 5 ml with 0.1 M HCl. To the extracts were added 1 ml 20% (w/v) $NaCO_3$ and 0.5 ml Folin-Ciocalteau reagent (Sigma). Solutions were shaken, incubated in a boiling water bath for 1 min and immediately chilled in ice. After cooling, solutions were diluted to 100 ml, filtered through qualitative filter paper, and recorded in spectrophotometer (Biospectro, São Paulo, Brazil) at 750 nm. A standard curve was prepared with pyrogallol. Data were expressed in µg pyrogallol equivalents per g extracted dry weight.

Tannin quantitation assay. Total soluble tannins were extracted as described (Rubanza et al., 2005). Air-dried samples (0.1 g) or fresh samples (0.2 g) were ground with liquid nitrogen and extracted in a mixture of 4 ml acetone:water (7:3) containing 1 g/l ascorbic acid and 2 ml methylene chloride for 30 min in a sonic bath. Then, the samples

were centrifuged at 1700 g for 15 min at 4°C. The aqueous phase was recovered and the pellet was re-extracted twice. The combined extracts were adjusted to 10 ml with distilled water.

Extracted tannins were assayed by the method of Hagerman and Butler (1978). To 0.5 ml aqueous extract, 1 ml protein solution (1 g/l bovine serum albumin in 0.2 M acetate buffer, pH 5.0 containing 0.17 M NaCl) was added. The solution was gently shaken and incubated at room temperature for 15 min. Next, the solution was centrifuged for 15 min at 5000 g. The supernatant was discarded and the pellet carefully washed with the protein solution. Then, the pellet was dissolved in 2 ml SDS-TEA (sodium dodecyl sulfate 1% and triethanolamine 5% in water) and 0.5 ml ferric reagent (10 mM ferric sulfide III in 0.01 mM HCl) was added. Between 15 and 30 min later, the solution absorbance was recorded at 510 nm in a spectrophotometer. A standard curve was prepared with tannic acid. Data were expressed per extracted dry weight.

Generalist herbivore bioassays. To test for deterrence effects of brachycerine, two broad spectrum model herbivores were used. *Spodoptera frugiperda* (Lepidoptera: Noctuidae) larvae were assayed following the "no choice" method proposed by van Dam et al. (2005), with minor modifications. Lettuce (*Lactuca sativa* L.) disks (2 cm diameter) were treated with methanol or a methanolic solution of brachycerine to a final concentration of 1, 2 or 3 mg brachycerine per g fresh weight. Four disks were placed on moistened filter paper inside a disposable Petri dish along with two *S. frugiperda* larvae at third instar. The samples (n=10) were kept overnight at 23°C, 16 h/day photoperiod, 75% relative humidity. Then, the insects were removed and the eaten disk area was measured.

Another test was done with the snail *Helix aspersa* L. using the method of Smith et al. (2001). Briefly, plastic boxes (30x15x12 cm) were divided into six chambers. Each one of the five chambers received a small water container and two individuals of *H. aspersa* weighing between 6 and 10 g. The snails were starved for 24 h in a growth cabinet at 18°C with 16 h/day photoperiod. Then, a pair of Whatman n°1 filter paper disks (5.5 cm diameter) was placed inside each chamber. Each disk pair was treated with one of the following solutions (0.25 ml per disk): methanol only, methanolic extract of field-grown *P. brachyceras* leaves, methanolic extract of field-grown *P. carthagenensis* leaves, or methanolic extract of *P. brachyceras* leaves from UV-C stressed cuttings. The UV-C treatment to induce brachycerine accumulation was performed as described (Gregianini et al., 2003). Snails were allowed to feed on the filter paper disks for 48 hours at 15°C and 16 h/day artificial light. Then, the disks were collected, dried at 50°C for one week and the remaining mass was recorded. The eaten sample amount mass was estimated by the difference between initial and remaining filter paper mass.

In situ localization of ROS in wounded leaves with and without brachycerine. H_2O_2 production upon wounding was assayed by the 3,3-diaminobenzidine (DAB) staining method (Thordal-Christensen et al., 1997). *Coleus blumei* Benth (white and green variegated) leaves were detached and the petioles were dipped in 1 mg/ml DAB solution for 8 hours. The solutions contained DAB alone or DAB plus brachycerine or ascorbic acid, both at 10 mM concentration. Damage was done with scissors and leaf samples were kept in solutions after wounding while polymer formation took place. The reaction was stopped after 15 minutes by bleaching leaf blades in boiling 95° (v/v) commercial ethanol for 10

min. Wounded areas were mounted on a microscopic slide in ethanol, observed and photographed under a stereomicroscope.

Alternatively, disks (5 mm diameter) from *C. blumei* leaves were obtained with a cork borer and placed in different petri dishes with a 0.5 cm film of the above solutions for 30 minutes in the dark. After 10 min of bleaching in boiling ethanol, disks were mounted in microscope slides and photographed under a stereomicroscope.

RESULTS

Wound-induced brachycerine and phenolics accumulation. A detailed time course analysis of brachycerine and phenolic compounds accumulation upon wounding was performed. Brachycerine accumulation increased in damaged samples and peaked 48 h after wounding (Fig. 1a), in agreement with previous observations (Gregianini et al., 2004). Induction was not statistically significant in wounded samples until 24 hours after treatment (Fig. 1a). Brachycerine contents from stem samples were not significantly changed by leaf wounding (Fig. 1b).

Total soluble phenolics accumulation was not changed by wounding treatments (Fig. 2). *P. brachyceras* leaf samples were also assayed for soluble tannins, but the extract had no protein precipitation activity. Protein-bound and fibre-bound condensed tannins could not be detected either. All tannin detection methods were also used with *Eucalyptus globulus* samples, yielding the expected positive results (data not shown).

Deterrence tests. Lettuce disks treated with authentic brachycerine were fed to *S. frugiperda* larvae. The test showed no deterrence effects for the alkaloid (Fig. 3a). Higher brachycerine concentrations were also tested (5 and 10 mg/g) with identical results (data not shown).

Filter paper disks were treated with *Psychotria* spp. extracts and offered to *Helix aspersa* snails. *P. brachyceras* extract from field-grown leaves and from UV-C stressed leaves contained 0.12 g/l (approx. 0.2 mM) and 0.6 g/l (approx. 1 mM) brachycerine respectively. None of the extracts deterred snail feeding (Fig. 3b). Surprisingly, filter paper disks treated with *P. carthagenensis* extract had less mass consumed. Further analysis revealed soluble tannin accumulation in *P. carthagenensis* leaf tissues, with a concentration of 2,70433 $\pm$ 0,14638 mg/g dry weight (n=6).

Wound-generated H_2O_2 quenching. Brachycerine treatment diminished DAB staining in wounded *Coleus* tissues (Fig 4). The lower H_2O_2 content on brachycerine-treated disks was apparent, although the decrease was not as pronounced as that caused by ascorbic acid, a widely used H_2O_2 scavenger. However, in the leaf uptake assay the effects of two antioxidant molecules were comparable.

DISCUSSION

Brachycerine concentration in leaves is regulated by wounding and jasmonates. It has previously been shown that this alkaloid is restricted to shoots, its biosynthesis is root-independent, and its maximum accumulation is observed in inflorescences (Gregianini et al., 2004). In the present investigation it became apparent that alkaloid production upon

wounding occurs in leaves, since brachycerine content in stems did not change significantly (Fig. 1). Wounding and jasmonate-induced accumulation can be found in herbivore-deterrent alkaloids, such as nicotine (Baldwin, 1988; Shoji et al., 2008) and gramine (Matsuo et al., 2001), and also in tannins (Peters and Constabel, 2002; Rossi et al., 2004) and other phenolics (Reyes et al., 2007; Andreotti et al., 2006). Since no tannins were found in *P. brachyceras* leaves and no alterations were detected in total phenolics upon wounding, damage-induced brachycerine was thought to play a role in defense responses of *P. brachyceras*. This putative protective role of brachycerine against herbivory was further suggested by the accumulation in reproductive organs, including inflorescences and fruit (Gregianini et al., 2004).

However, the alkaloid failed to deter both *S. frugiperda* and *H. aspersa* feeding, even at concentrations higher than the usually found in leaves. This result does not support the defense hypothesis. Toxic alkaloids such as nicotine display a whole-plant induction pattern by wounding (Baldwin et al., 1994). Nonetheless, wound-induced brachycerine accumulation is restricted to the damaged site (Gregianini et al., 2004).

A closer look at field-grown *P. brachyceras* shows that these plants are very scarcely predated. Both *P. brachyceras* and *P. carthagenensis* leaves offered to the herbivores used in the experiments were left undamaged. Field-grown *P. carthagenensis* plants, however, are very often found severely damaged, apparently by herbivore chewing. The exact herbivore species which feed on *P. carthagenensis* are currently unknown, but their presence could explain the observed tannin accumulation in leaves of this species. On the other hand, as suggested by the deterrence experiments, *P. brachyceras* shows no obvious chemical defenses, although it may rely on protein-based deterrence mechanisms, such as proteinase inhibitors.

Taken together, the observations suggest another type of function for brachycerine. The limited damage/herbivory stress to *P. brachyceras* individuals may have led to the acquisition of tolerance rather than defense mechanisms. Plants can withstand 10 to 25% of shoot loss without significant costs to reproductive fitness (Strauss and Agrawal, 1999). Mechanical damage elicits local production of reactive oxygen species (Orozco-Cardenas and Ryan, 1999), and brachycerine, a highly antioxidant compound [Gregianini et al., 2003; Nascimento et al., 2007; Porto et al., 2009 (unpublished)], may help detoxify wound-generated oxidative species, protecting cellular structures and avoiding abscission of the damaged organ. To investigate brachycerine functions on wound-induced oxidative burst, a hydrogen-peroxide quenching bioassay was developed using 3,3-diaminobenzidine, a widely used compound for histochemical staining of *in vivo* produced H_2O_2 (Thordal-Christensen *et al.,* 1997). Clearly, brachycerine inhibited the brown-coloured H_2O_2-driven DAB polymerization at least to some extent, albeit less intensively than ascorbic acid, a widely used H_2O_2 scavenger, in the disk assay.

Brachycerine may participate on protecting tissues from wound-induced oxidative stress. The data is in agreement with previously described bioassays, in which brachycerine protected catalase-silenced mutant yeast cells from H_2O_2 damage (Nascimento et al., 2007). In this regard, brachycerine may perform partly overlapping functions in wounding and UV stress responses [Gregianini et al., 2003; Porto et al., 2009 (unpublished)], acting as an inducible general antioxidant agent to limit and protect against oxidative stress. However, since hydrogen peroxide production often peaks between 4 and 6 hours after wounding (Orozco-Cardenas and Ryan, 1999) and significant brachycerine induction is seen only 24 h after damage, the alkaloid may perform other roles on wound stress besides detoxification

of oxidative species, or the later increase may prepare for possible further oxidative stress events. In conclusion, data indicate that brachycerine is not a herbivore deterrent, but rather an indirect chemical defense, modulating oxidative stress derived from mechanical damage.

REFERENCES

ANDREOTTI, C., COSTA, G., TREUTTER, D. 2006. Composition of phenolic compounds in pear leaves as affected by genetics, ontogenesis and the environment. *Sci. Hortic.-Amsterdam* 109:130-137.

ARIMURA, G., OZAWA, R., SHIMODA, T., NISHIOKA, T., BOLAND, W., TAKABAYASHI, J. 2000. Herbivory-induced volatiles elicit defense genes in lima bean leaves. *Nature* 406: 512-515.

BALDWIN, I. T. 1988. Short-term damage-induced increases in tobacco alkaloids protect plants. *Oecologia* 75: 367-370.

BALDWIN, I. T., SCHMELZ, E. A., OHNMEISS, T. E. 1994. Wound-induced changes in root and shoot jasmonic acid pools correlate with induced nicotine synthesis in *Nicotiana sylvestris* spegazzini and comes. *J. Chem. Ecol.* 20(8): 2139-2153.

BOUDET, A. 2007. Evolution and current status of research in phenolic compounds. *Phytochemistry* 68: 2722-2735.

DE LUCA, V. and LAFLAMME, P. 2001. The expanding universe of alkaloid biosynthesis. *Curr. Op. Plant Biol.* 4: 225-233.

DESPRÉS, L., DAVID, J., GALLET, C. 2007. The evolutionary ecology of insect resistance to plant chemicals. *Trends Ecol Evol* 22(6): 298-307.

DIXON, R. A. and PAIVA, N. L. 1995. Stress-induced phenylpropanoid metabolism. *Plant Cell*, 7: 1085-1097.

EHRLICH, P. R. and RAVEN, P. H. 1964. Butterflies and plants: a study in coevolution. *Evolution* 18: 586-608.

FETT-NETO, A. G., TEIXEIRA, S. L., SILVA, E. A. M., SANT´ANNA, R. S. 1992. Biochemical and morphological changes during in vitro rhizogenesis in cuttings of *Sequoia sempervirens* (D.Don) Endl. *J. Plant Physiol.* 140: 720-728.

GATEHOUSE, J. A. 2002. Plant resistance towards insect herbivores: a dynamic interaction. *New Phytol.* 156: 145-169.

GOULD, K. S., MCKELVIE, J., MARKHAM, K. R. 2002. Do anthocyanins function as antioxidant in leaves? Imaging of H_2O_2 in red and green leaves after mechanical injury. *Plant Cell Environ.* 25: 1261-1269.

GREGIANINI, T. S.; SILVEIRA, V. C.; PORTO, D. D.; KERBER, V. A.; HENRIQUES, A. T.; FETT-NETO, A. G. 2003. The alkaloid brachycerine is induced by ultraviolet radiation and is a singlet oxygen quencher. *Photochem. Photobiol.* 78(5): 470-474.

GREGIANINI, T. S.; PORTO, D. D.; NASCIMENTO, N. C.; FETT, J. P.; HENRIQUES, A. T.; FETT-NETO, A. G. 2004. Environmental and ontogenetic control of accumulation of brachycerine, a bioactive indole alkaloid from *Psychotria brachyceras. J. Chem. Ecol.* 30(10): 2023-2036.

KERBER, V. A., GREGIANINI, T. S., PARANHOS, J., SCHWAMBACH, J., FARIAS, F., FETT, J. P., FETT-NETO, A. G., ZUANAZZI, J. A. S., QUIRION, J., ELISABETSKY, E., HENRIQUES, A. T. 2001. Brachycerine, a novel monoterpene indole alkaloid from *Psychotria brachyceras. J. Nat. Prod.* 64(5): 677-679.

KESSLER, A. and BALDWIN, I. T. 2002. Plant responses to insect herbivory: the emerging molecular analysis. *Annu. Rev. Plant Biol.* 53: 299-328.

LEAL, M. B. and ELISABETSKY, E. 1996. Absence of alkaloids in *Psychotria carthagenensis* Jacq. (Rubiaceae). *J. Ethnopharmacol.* 54: 37-40.

MAKKAR, H. P. S. 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects on feeding tannin-rich feeds. *Small Ruminant Res* 49: 241-256.

MATSUO, H., TANIGUCHI, K., HIRAMOTO, T., YAMADA, T., ICHINOSE, Y., TOYODA, K., TAKEDA, K., SHIRAISHI, T. 2001. Gramine increase associated with rapid and transient systemic resistance in barley seedlings induced by mechanical and biological stress. *Plant Cell Physiol.* 42(10): 1103-1111.

MURASHIGE, T., and SKOOG, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plantarum* 15: 709-714.

NASCIMENTO, N. C., FRAGOSO, V., MOURA, D. J., SILVA, A. C. R., FETT-NETO, A. G., SAFFI, J. 2007. Antioxidant and antimutagenic effects of the crude foliar extract and the alkaloid Brachycerine of *Psychotria brachyceras. Environ. Mol. Mutagen.* 48(9): 728-734.

OROZCO-CARDENAS, M. and RYAN, C. 1999. Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. *Proc. Natl. Acad. Sci. USA*. 96: 6553-6557.

PETERS, D. J. and CONSTABEL, C. P. 2002. Molecular analysis of herbivore-induced condensed tannin synthesis: cloning and expression of dihydroflavonol reductase from trembling aspen (*Populus tremuloides*). *Plant J.* 32: 701-712.

REYES, L. F., VILLAREAL, J. E., CISNEROS-ZEVALLOS, L. 2007. The increase in antioxidant capacity after wounding depends on the type of fruit and vegetable tissue. *Food Chem.* 101: 1254-1262.

ROSSI, A. M., STILING, P., MOON, D. C., CATTELL, M. V., DRAKE, B. G. 2004. Induced defensive response of myrtile oak to foliar insect herbivory in ambient and elevated CO_2. *J. Chem. Ecol.* 30(6): 1143-1152.

RUBANZA, C. D. K.; SHEM, M. N.; OTSYINA, R.; BAKENGESA, S. S.; ICHINOHE, T.; FUJIHARA, T. 2005. Polyphenolics and tannin effect on in vitro digestibility of selected Acacia species leaves. *Anim. Feed Sci. and Technol.* 119: 129-142.

SHOJI, T., OGAWA, T., HASHIMOTO, T. 2008. Jasmonate-induced nicotine formation in tobacco is mediated by tobacco *COI1* and *JAZ* genes. *Plant Cell Physiol.* 49(7): 1003-1012.

SICILIANO, T., DE LEO, M., BADER, A., DE TOMMASI, N., VRIELING, K., BRAÇA, A., MORELLI, I. 2005. Pyrrolizidine alkaloids from *Anchusta strigosa* and their antifeedant activity. *Phytochemistry* 66: 1593-1600.

SMITH, D. B., RODDICK, J. G., JONES, J. L. 2001. Synergism between the potato glycoalkaloids α-chaconine and α-solanine in inhibition of snail feeding. *Phytochemistry* 57: 229-234.

STOWE, K. A., MARQUIS, R. J., HOCHWENDER, C. G., SIMMS, E. L. 2000. The evolutionary ecology of tolerance to consumer damage. *Annu. Rev. Ecol. Syst. 31:* 565-95.

STRAUSS, S. Y. and AGRAWAL, A. A. 1999. The ecology and evolution of plant tolerance to herbivory. *Trees* 14(5): 179-185.

THORDAL-CHRISTENSEN, H.; ZHANG, Z.; WEI, Y.; COLLINGE, D. B. 1997. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during barley-powdery mildew interaction. *Plant Journal 11(6):* 1187-1194.

TURLINGS, T. C. J., LOUGHRIN, J. H., MCCALL, P. J., RÖSE, U. S. R., LEWIS, W. J., TUMLINSON, J. H. 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proc. Natl. Acad. Sci. USA.* 92: 4169-4174.

VAN DAM, N. M., VUISTER, L. W. M., BERGSHOEFF, C., DE VOS, H., VAN DER MEIJDEN, E. 1995. The "raison d'être" of pyrrolizidine alkaloids in *Cynoglossum officinale:* deterrent effects against generalist herbivores. *J. Chem. Ecol.* 21(5): 507-523.

WASTERNACK, C. 2007. Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Ann. Bot.* 100: 681-697.

WITTSTOCK, U. and GERSHENZON, J. 2002. Constitutive plant toxins and their role against herbivores and pathogens. *Curr. Op. Plant Biol.* 5: 300-307.

XIAO, Z., HARRIS, M. K., ZHANG, Z. 2007. Acorn defenses to herbivory from insects: Implication for the joint evolution of resistance, tolerance and escape. *Forest Ecol. Manag.* 238: 302-308.

Figure 1: Brachycerine content of *P. brachyceras* cuttings after mechanical damage in leaves. Brachycerine concentrations were assayed in leaves (A) and stems (B) of treated and untreated cuttings. The asterisk indicates statistical difference by *t* test for $p \leq 0.05$. Bars on top of columns are standard deviations.

Figure 2: Total soluble phenolics content in *P. brachyceras* leaves after wounding. Bars on top of columns are standard deviations.

Figure 3: Deterrency bioassays. A: Lettuce disk area eaten by *S. frugiperda* larvae. Disks were treated with MeOH only or with brachycerine methanolic solutions to the indicated concentrations over fresh weight. B: *H. aspersa* bioassay. Filter paper disks were treated with MeOH only or with methanolic extracts of *Psychotria* species and offered to snails for 48 h. The asterisk indicates statistical difference by Tukey test for $p \leq 0.05$. Bars on top of columns are standard deviations.

Figure 4: DAB staining bioassay. Disk incubation: *Coleus blumei* leaf disks were incubated with DAB solutions. The influence of brachycerine and ascorbic acid on color formation was tested in co-incubation tests with DAB. Leaf uptake: petioles of detached *C. blumei*. leaves were immersed in DAB solutions with or without brachycerine or ascorbic acid, and color formation was followed after damage with scissors. In both types of assays, darker brown staining indicates higher formation of peroxide upon wounding.

Figure 1a

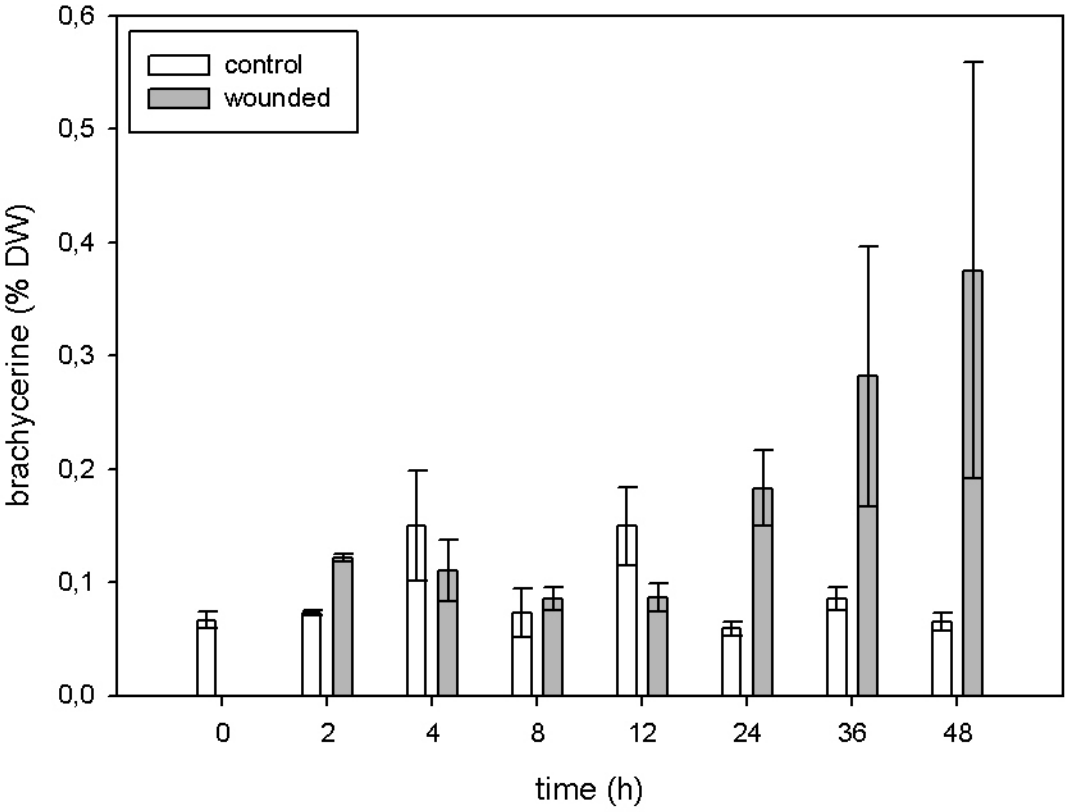

control
wounded
brachycerine (% DW)
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0
2
4
8
12
24
36
48
time (h)

Figure 1b

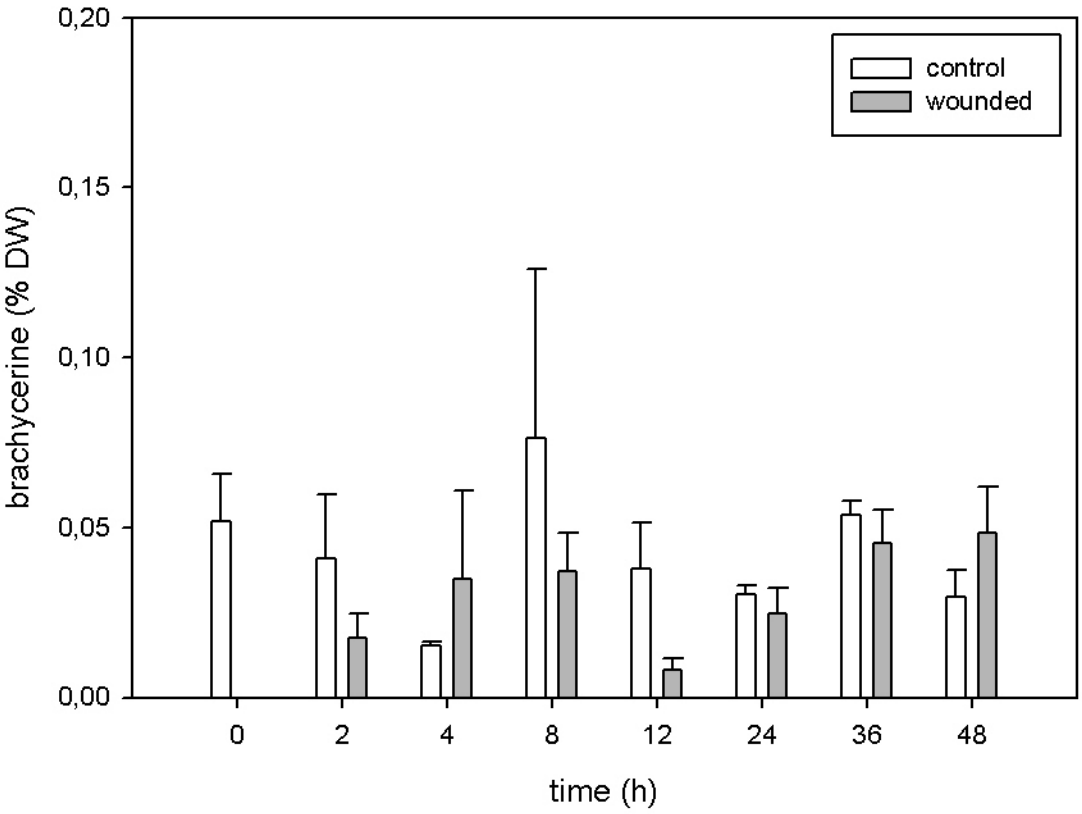

0,20
0,15
0,10
0,05
0,00
brachycerine (% DW)
control
wounded
0
2
4
8
12
24
36
48
time (h)

Figure 2

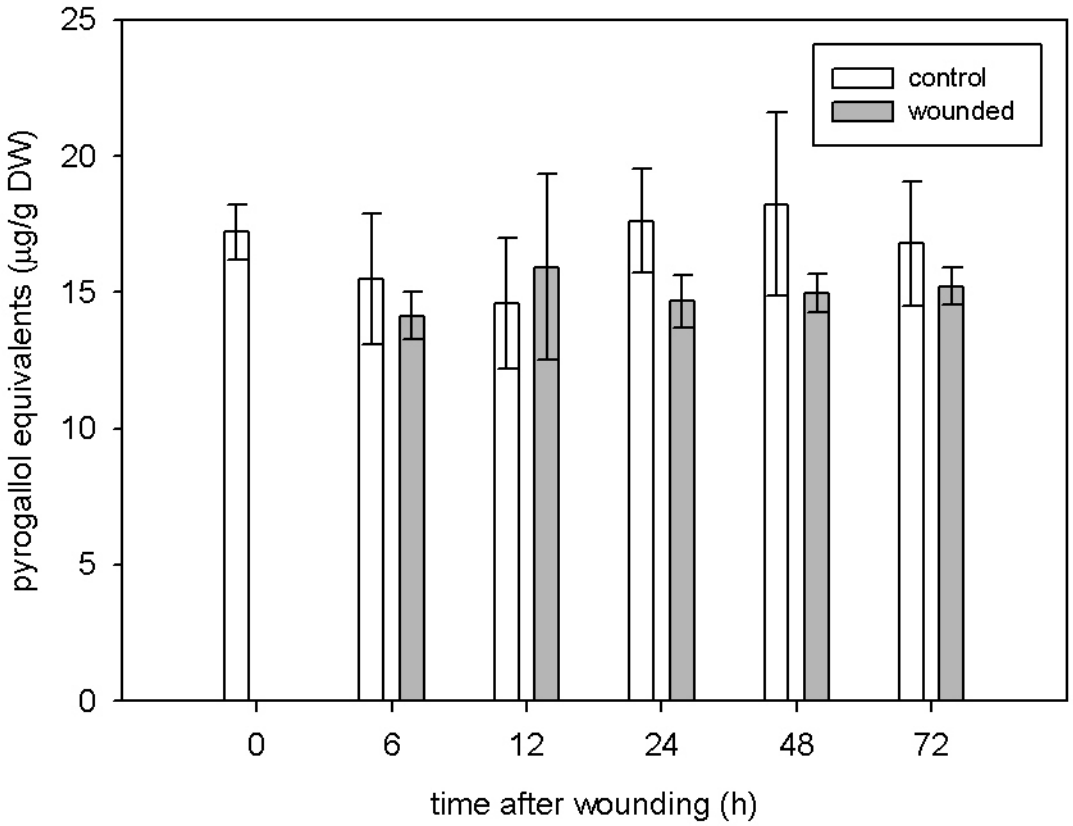

25
20
15
10
5
0
pyrogallol equivalents (µg/g DW)
control
wounded
0
6
12
24
48
72
time after wounding (h)

Figure 3a

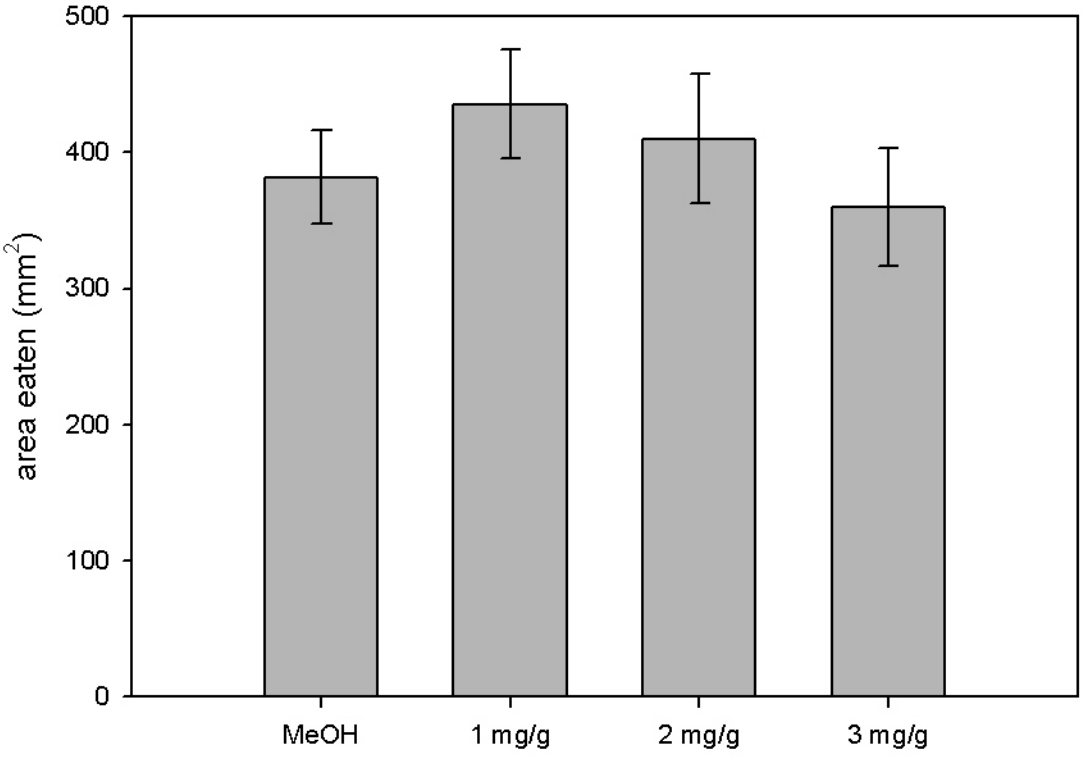


Figure 3b

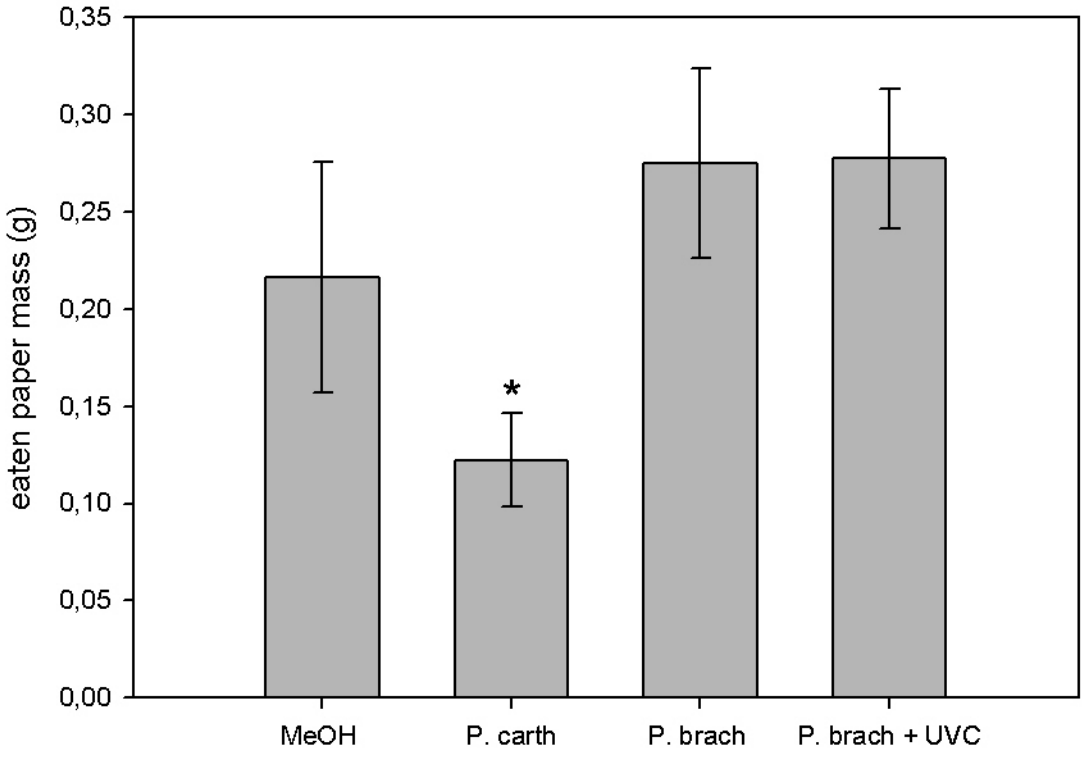
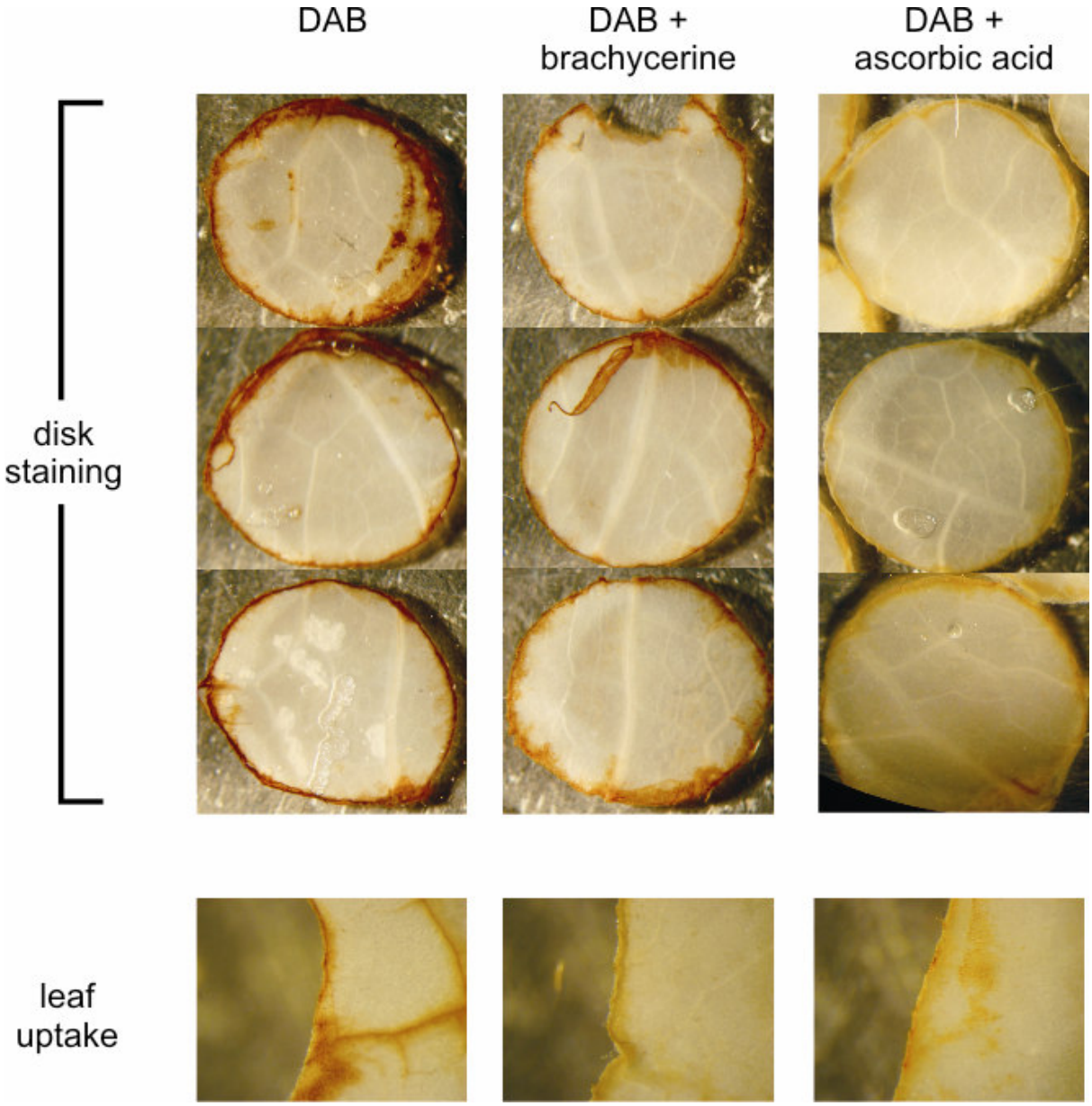

0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
eaten paper mass (g)
*
MeOH
P. carth
P. brach
P. brach + UVC

Figure 4


DAB
DAB +
brachycerine
DAB +
ascorbic acid
disk
staining
leaf
uptake

PARTE II : RADIAÇÃO ULTRAVIOLETA

MANUSCRITO 2: Ultraviolet radiation tolerance mediated by brachycerine, a bioactive monoterpene-indole alkaloid from Psychotria brachyceras.

Artigo submetido ao Periódico Tree Physiology

Ultraviolet radiation tolerance mediated by brachycerine, a bioactive monoterpene-indole alkaloid from *Psychotria brachyceras*

Diogo Denardi Porto[1], Hélio Nitta Matsuura[2], Camilo Pedrollo[2], Denilson Almeida dos Santos[2], Amélia Terezinha Henriques[3], Luis Mauro Gonçalves Rosa[4], Arthur Germano Fett-Neto[1,2] *

[1]Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, CP 15005, Porto Alegre, Rio Grande do Sul, 91501-610, Brazil.

[2]Departamento de Botânica, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

[3]Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

[4]Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

* corresponding author: Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, CP 15005, Porto Alegre, Rio Grande do Sul, 91501-610, Brazil, Phone 55-51-33087642, Fax: 55-51-3308-7309, Email fettneto@cbiot.ufrgs.br.

Date of submission: February , 2009.

This manuscript contains 7 figures and no tables.

Running title: Ultraviolet tolerance mediated by alkaloid in *Psychotria brachyceras*

ABSTRACT

Plants developed several strategies to cope with ultraviolet radiation (UV) stress, including accumulation of secondary metabolites, with a major role played by flavonoids. The unique alkaloid brachycerine of *Psychotria brachyceras* (Rubiaceae) is induced by leaf exposure to UV and is a potent antioxidant, so it is likely to play a protecting role against this radiation. A closely related and co-ocurring species *P. carthagenensis* does not have alkaloids and was used as a comparison with *P. brachyceras* in UV-B exposure experiments. *P. brachyceras* leaves were very tolerant to high UV-B doses when compared to *P. carthagenensis*. The alkaloid was found in the epidermis and mesophyll. Well known leaf defenses against UV, such as flavonoids and cuticular waxes, were not markedly different between *Psychotria* species or were less expressed in *P. brachyceras*, so a role for brachycerine was apparent. Brachycerine was an effective *in vitro* superoxide scavenger and, when applied to the surface of *P. carthagenensis* leaves, significantly improved UV-B tolerance. Profiles of brachycerine extracted from *P. carthagenensis* leaves after UV exposure showed partial degradation of the alkaloid, indicating its involvement in chemical quenching of reactive oxygen species. The alkaloid brachycerine is a major component of UV protection in *P. brachyceras* and acts as both sunscreen and antioxidant.

Keywords: alkaloid, brachycerine, *Psychotria brachyceras, Psychotria carthagenensis*, secondary metabolites, superoxide, ultraviolet.

INTRODUCTION

Depletion of the ozone-layer is increasing the incidence of ultraviolet-B radiation (UV-B, 280-315 nm) on the Earth's surface, particularly at high latitudes. The UV-B waveband is absorbed by a variety of cellular components and causes multilevel oxidative stress (Hollósy, 2002). Among the damaged structures are DNA, proteins and photosynthetic pigments.

In the course of evolution, plants developed several adaptations to tolerate UV-B imposed stress (Hollósy, 2002, Frohnmeyer and Staiger, 2003; Jansen *et al*., 1998), comprising structural, enzymatic and chemical defenses. Leaf cuticles are the first barrier to UV penetration, and their morphological (shape) and chemical (composition and amount of epicuticular waxes) characteristics modulate the UV reflectance and absorbance of plant leaves (Grant *et al*., 2003; Krauss *et al*., 1997). UV-protection mechanisms also include production and accumulation of secondary metabolites, with some being vital for the acclimation to the environment. UV exposure induces phenylpropanoid metabolism (Dixon and Paiva, 1995), particularly flavonoids (Harborne and Williams, 2000), including anthocyanins, which may protect against UV-B (Mori *et al*., 2005; Kytridis and Manetas, 2006).

Alkaloids are small molecules containing nitrogen, often derived from amino acids (De Luca and Laflamme, 2001). Many alkaloids show medicinal properties, and various currently used pharmaceuticals belong to this class of metabolites or are modified versions of these. Production and storage of these substances by plants is well documented as defense responses to herbivore and pathogen attacks (Siciliano *et al.,*

2005; Wittstock and Gershenzon, 2002; Gatehouse, 2002); however, relatively little is known about their participation in UV responses.

Rubiaceae is among the botanical families that accumulate alkaloids. The genus *Psychotria* comprises more than a thousand species mostly distributed in the tropics. South American species of this genus have been found to accumulate indole alkaloids, some of which with pharmacological properties (Pasquali *et al*., 2006). *Psychotria brachyceras* and *P. carthagenensis* are understorey woody species that grow in subtropical semi-deciduous forests of Southern Brazil. Whereas the former contains alkaloids, the later is devoid of these metabolites (Leal and Elisabetsky, 1996).

A major leaf-accumulating alkaloid, brachycerine (Fig. 1), was described in *Psychotria brachyceras* (Kerber *et al*., 2001), and to date it has not been found in any other species. Brachycerine is a monoterpene-indole alkaloid with an unusual structure, having a terpene moiety apparently derived from epiloganin and not from the conventional secologanin, besides retaining a glucose residue, normally absent in most of the known indole alkaloids. Brachycerine is absent in roots and accumulates in leaves, stems, flowers and fruit; independent on the presence of roots, leaf alkaloid concentration in cuttings increases upon elicitation with mechanical damage (Gregianini *et al.,* 2004) and UV radiation (Gregianini *et al*., 2003). A seasonal pattern of brachycerine accumulation peaking in the Fall was observed, possibly due to increased irradiance in the understorey, as several upper storey tree species lose their leaves at that time (Gregianini *et al*., 2004). Several properties of brachycerine support a role in UV tolerance: absorption at UV-B wavelengths and antioxidant activities against singlet oxygen (Gregianini *et al*., 2003) and hydroxyl radicals (Nascimento *et al*., 2007).

The objective of this work was to determine if brachycerine plays a relevant role in protecting against UV-B stress. To that end, the accumulation profiles of well established UV protectant metabolites in control and high UV-B conditions were followed in time course experiments in relation to UV-B induced damage to chlorophylls of both *P. brachyceras* and *P. carthagenensis*. Leaf anatomical structure was also compared between the species. Antioxidant activity of brachycerine towards superoxide anion was tested *in vitro*, as well as the UV-protective effects of the alkaloid in an *in vivo* assay with *P. carthagenensis* treated with brachycerine. Possible roles for brachycerine on UV protection are discussed.

MATERIALS AND METHODS

Plant material

Field-grown *P. brachyceras* Mull. Arg. and *P. carthagenensis* Jacq. adult plants located at the Biological Reserve of Morro Santana – campus do Vale - UFRGS, Porto Alegre, Rio Grande do Sul, Brazil were used in the experiments. Voucher specimens are deposited at UFRGS ICN herbarium (7899 and 7901, respectively). Tip cuttings (20 cm long, with four to six leaves per cutting) were harvested and incubated in nutrient solution of MS salts (Murashige and Skoog, 1962) at 10% strength for ten days before experimental procedures started. The growth room was maintained at 25±3°C and the cuttings received 16h day^{-1} of 73 μmol m^{-2} s^{-1} photosynthetically active radiation (provided by cool white fluorescent lamps).

Cuticular waxes content

Total cuticular waxes were assayed by the method of Pilon *et al*. (1999) with minor modifications. Four to six leaves were immersed in 150 ml chloroform-methanol (2 : 1, v : v) and gently moved around the solution for 1 min. The extract was filtered in Whatman n°1 filter paper and evaporated to near 1 ml. Extract was then quantitatively transferred to previously weighed microcentrifuge tubes and evaporated to dryness in a SpeedVac system (Savant, USA). Tubes were then weighed again and the difference was calculated. Data was expressed per leaf area. Leaf area was measured by drawing models of the leaves on paper, weighing these and comparing to weight of known areas of the same paper using an analytical balance.

Expanded leaves of the outer crown of field-grown trees of each *Psychotria* species (2 leaves from each one of 5 individuals with similar light exposure in the same area of the understorey forest) were harvested to examine mesophyll structure. Transversal hand sections of fresh leaves were prepared with steel blades, mounted on slide and stained with Sudan III (0.1% w/v in one part of 96% ethanol to two parts of glycerol) to mark the cuticles. Sections were photographed in a photomicroscope

UV treatments

Cuttings were UV-stressed in cabinets equipped with an ultraviolet lamp (UVB-313EL, Q-Lab, Ohio, USA, max. wavelength 313 nm) placed in parallel with a cool white-light fluorescent lamp and 45 cm above cuttings, similar to previously described experiments (Gregianini *et al*., 2003). UV-treated cuttings were exposed to white light (as described above) supplemented with 68.89 KJ m^{-2} day^{-1} UV-B/UV-A radiation (39.37 KJ m^{-2} day^{-1}

biologically effective radiation [Caldwell, 1971]). Control cuttings remained under white light only (16 h day^{-1}). Samples, harvested at the day of treatment application and after three, six and nine days of exposure, were frozen in liquid nitrogen and stored at -20°C until analyses.

Brachycerine extraction and quantitative analyses

Alkaloid extraction and analysis were performed essentially as described (Gregianini *et al.,* 2003). Shortly, 150 mg of liquid nitrogen-powdered tissue were extracted in 1 ml methanol by 30 min sonication. The supernatant was recovered and analyzed in a Perkin-Elmer S200 HPLC. Chromatography was performed in a C-8 reverse phase Waters column using a gradient with methanol-water-trifluoroacetic acid. Quantification was performed using external standard curves made with authentic brachycerine isolated from leaves (Kerber *et al.*, 2001). Brachycerine content was expressed on a dry weight basis.

Flavonoid and chlorophyll analysis

Total flavonoid content was estimated spectrophotometrically using the method of Zhishen *et al.* (1999) with minor modifications. In a dimmed-light room, 200 mg of N_2-ground fresh tissue (n=5) were extracted in 5 ml 95% ethanol by 30 min ultrasonication. The suspension was centrifuged and 1 ml extract was used in the reaction as follows: the extract was diluted to 5 ml with water, and 0.3 ml of 5% $NaNO_2$ was added. After 5 min incubation, 0.3 ml of 10% $AlCl_3$ was added and the solution was incubated for another 6 min. The reaction was then stopped with 2 ml of 1 M NaOH and the total volume was adjusted to 10 ml. Absorbance at 510 nm was measured with a BioSpectro

spectrophotometer (São Paulo, Brazil). A calibration curve was prepared with quercetin (Sigma, USA) as standard flavonoid, and total flavonoid content was expressed as quercetin equivalent per dry weight basis.

Total anthocyanin content was estimated by the method of Chatterjee *et al.,* 2006. Samples (n=5) were ground and extracted in 2 ml acid methanol (MeOH-conc HCl 99 : 1, v : v) overnight, in the dark, under constant shaking. The samples were then filtered and volume was made again to 2 ml. Chloroform (2 ml) was added and samples were mixed. Double distilled water (1 ml) was then added and samples were vigorously mixed for 10 s. After phase separation, absorbance of the aqueous (upper) phase was recorded at 530 nm in spectrophotometer. Anthocyanin content was expressed as absorbance per extracted dry weight.

Total chlorophylls were assayed by the method of Ross (1974). Leaf disks (1 cm diameter, 3 disks per sample, n=5) were cut and ground with liquid nitrogen in the dark and extracted in acetone 85% by sonication. After centrifugation at 10 000 *g* for 10 min, the absorbance of the extract at 645 and 663 nm was recorded. The cell debris was dried for a week at 60°C and the extracted dry weight was determined.

Brachycerine isolation

Authentic brachycerine was isolated from plant leaves as described (Kerber *et al.,* 2001). Air-dried leaves (20-25 °C in the shade for 2 weeks) were homogenized in a waring blender with commercial ethanol and macerated for three weeks in the dark. Weekly, the extract was recovered by filtration and fresh ethanol was added to plant debris to maximize yield. The combined extracts were evaporated to dryness, resuspended in 0.1

M HCl and washed with methylene chloride. The pH of the aqueous extract was then adjusted to 10.0 with ammonium hydroxide and the alkaloids were extracted in methylene chloride with a soxhlet. Brachycerine was separated by flash column chromatography using a gradient with chloroform-methanol.

In vivo chlorophyll protection assay

P. carthagenensis fully expanded leaves from the outer crown of a field-grown adult plant were harvested and washed. Disks (2 cm diameter) were cut and treated with 16 µl of brachycerine solution (1 or 10 g l^{-1}) or methanol using a soft brush. Treated disks were placed on moistened filter paper under white light supplemented with UV-B/UV-A radiation for 4 days (130.90 KJ m^{-2} day^{-1}, 47.14 KJ m^{-2} day^{-1} biologically effective radiation [Caldwell, 1971]). A glass plate (50 mm thick) was placed above control disks to filter out UV-B and most UV-A wavelengths. Chlorophylls were extracted from two disks per sample (ca. 150 mg) in 1.5 ml chilled 85% acetone with a tissue homogenizer (PowerGen 125, Fisher). Samples (n=3) were sonicated in ice bath for 15 min and centrifuged at 13 000 *g* for 10 min at 4°C. Pellet was re-extracted twice and final volume was made up to 5 ml. Total chlorophylls were estimated at 645 and 663 nm (Ross, 1974).

Superoxide scavenging activity

The *in vitro* assay was based on the method of Zhishen *et al*. (1999). Authenthic brachycerine was diluted in methanol-water (1 : 2, v : v) and added to reaction medium to a final concentration of 0.5 mM. The reaction medium consisted of 50 mM phosphate buffer (pH 7.8) containing 3 µM riboflavine, 10 mM methionine and 0.1 mM

nitrobluetetrazolium. Solutions (n=3) were exposed to white light for 10 minutes, and the blue formazan produced was measured at 560 nm in spectrophotometer. Controls without brachycerine (negative) or with Trolox 0.5 mM (positive) instead of the alkaloid diluted in the same solvent were tested under the same conditions.

Epidermis isolation

Epidermal strips were manually obtained from freshly harvested shoots with young leaves (about two-thirds expanded and of lighter green color) kept in water. Strips from both epidermal faces combined were removed using a razor blade and histological tweezers under a stereomicroscope. Immediately after removal, strips were washed in water, kept on ice, weighed and extracted as described above. Integrity and purity of epidermis were routinely monitored every few strips using a microscope to verify the absence of significant contamination with residues of mesophyll cells.

Statistical analysis. Experiments were independently performed at least twice with 3 to 5 replicates per treatment. Means were subjected to Fisher's One-way Analysis of Variance followed by Tukey test or to Student's *t* test, whenever applicable, using the SPSS v. 13 software. Only confidence intervals of 95% or higher were considered.

RESULTS

Brachycerine accumulation is induced by high UV stress

Exposure of *P. brachyceras* cuttings to UV radiation caused a significant increase in

brachycerine content (Fig. 2). Although significant increases in brachycerine content in UV-treated cuttings were only detected at day 9, a time-dependent increase trend was observed in the previous time points.

The two *Psychotria* species differ in chlorophyll loss upon UV stress

The response of total chlorophylls of *P. brachyceras* and *P. carthagenensis* to UV radiation was evaluated. *P. carthagenensis* seemed to be much less tolerant to UV radiation in comparison to *P. brachyceras* (Fig. 3). A slight decrease in chlorophyll content and chlorophyll *a*/*b* ratio was seen at day six in the latter (Fig 3a,b). On the other hand, *P. carthagenensis* showed severe injuries, and samples could not be harvested at day 9. Chlorophyll contents dropped as early as day 3 (Fig 3c), and chlorophyll *a/b* ratio decreased at day 6 (Fig 3d).

The two *Psychotria* species have distinct cuticle organization, cuticular wax layer contents and properties

The marked differences in tolerance to UV radiation between the species led to questions on possible protective mechanisms responsible for those differences. Cross sections of leaves of both species followed by Sudan III staining revealed that typical cuticles had different shapes. *P. carthagenensis* often showed globular waxy structures (Fig. 4b), whereas *P. brachyceras* showed a more uniform thin layer in the adaxial face of leaves (Fig. 4a). Quantification of total waxes showed a two-fold higher content in the sensitive

species (Fig 4c).

In order to test for differences in wax transmittance that could contribute to increased UV tolerance of *P. brachyceras*, extracted epicuticular waxes were diluted in HPLC grade methanol (0.3 mg ml^{-1}) and the UV transmittance was recorded in spectrophotometer. Means (n=4) at 300 nm were not statistically different between the species (64.07 $\pm$ 2.13% for *P. brachyceras* and 57.7 $\pm$ 2.6% for *P. carthagenensis*); however, at 280 nm *P. carthagenensis* waxes (39.17 $\pm$ 4.7%) were significantly less transparent to UV when compared to *P. brachyceras* (54.77 $\pm$ 2.54%).

Leaf structure differed between species.

Leaf thickness of expanded leaves was similar between *P. brachyceras* and *P. carthagenensis* (183.8 ± 20.9 μm and 178.8 ± 15.8 μm, respectively). Leaves of *P. carthagenensis* had typical dorsiventral palisade structure, whereas *P. brachyceras* showed a two palisade organization, with a thinner and less densely packed chrorophyll parenchyma in the abaxial side in addition to a palisade in the adaxial face (Fig 4a).

Anthocyanin, but not total flavonoid content of *P. brachyceras* is UV-regulated

None of the *Psychotria* species showed significant changes in total flavonoid content upon UV stress (Fig. 5a). Total flavonoid content in *P. carthagenensis* was consistently higher than that of *P. brachyceras*. Anthocyanins of *P. carthagenensis* did not change

upon UV-treatment, but *P. brachyceras* showed UV-induced anthocyanin accumulation (Fig. 5b). It is noteworthy that initial concentrations of anthocyanins in *P. carthagenensis* leaves were considerably higher than those in *P. brachyceras*, even after UV-induction of leaves in the latter.

Brachycerine treatment prevents chlorophyll loss upon UV stress in *P. carthagenensis*

The biochemical and structural properties of the two *Psychotria* species together with the marked differences in UV sensitivity suggest an important role of brachycerine in UV tolerance. In an attempt to test the protective role of brachycerine *in vivo*, leaf disks from *P. carthagenensis* were treated with brachycerine in two different concentrations (simulating the average basal and UV-induced contents in *P. brachyceras* leaves) and exposed to strong UV irradiation stress. Four days later, brachycerine treated samples showed higher total chlorophyll content and chlorophyll *a/b* ratio when compared to controls (Fig. 6a,b). Total chlorophyll content increased with higher concentration of brachycerine. Therefore, brachycerine application on *P. carthagenensis* leaves was able to confer an UV tolerant phenotype to this species in a dose dependent manner.

Brachycerine is a superoxide scavenger *in vitro*

Besides sunscreening, UV-protection can be achieved by detoxification of oxidative stress derived from UV exposure. To test whether brachycerine is a scavenger of superoxide anion, an *in vitro* assay was performed. The alkaloid showed strong

antioxidant activity (Fig. 7), but still less effective than Trolox, an analog of tocopherol, used as positive control.

Brachycerine accumulates in the leaf epidermis

Brachycerine present in young leaves of *P. brachyceras* was found in the epidermal layers. Epidermal strips yielded approximately 0.36 ± 0.08 % brachycerine on an extracted dry weight basis (EDW) (mean ± S.E.), whereas entire leaf blades had 0.33 ± 0.15 % EDW of brachycerine. Therefore, at least part of the alkaloid is located in the external tissues of the leaf.

DISCUSSION

Plant responses to increased UV-B seem to depend on the intensity of the incoming radiation within this range of the spectrum (Brosché and Strid, 2003). In this study, a strong UV-B dose was irradiated on cuttings, yielding a four-fold increase in alkaloid content of treated cuttings. In a previous experiment, *P. brachyceras* cuttings were exposed to radiation from a lower intensity UV-B emitting source, and brachycerine contents increased two-fold in comparison to control values (Gregianini *et al*., 2003).

Reports on UV-induction of alkaloid biosynthesis are scarce. The model plant *Catharanthus roseus* synthesizes over 100 different monoterpene indole alkaloids, several of them upregulated by UV-B treatment (Ouwerkerk *et al*., 1999; Ramani and

Jayabaskaran, 2008). This accumulation seems to be a consequence of higher expression of the tryptophan decarboxylase gene, which encodes the enzyme that produces tryptamine, the indole precursor to alkaloid synthesis (Ouwerkerk *et al*., 1999).

UV-stressed cuttings of both *Psychotria* species showed reduced total chlorophylls and chlorophyll *a/b* ratio. These responses have been reported for several UV-B stressed plants (Sprtová *et al*., 1999; Smith *et al*., 2000) but it is not ubiquitous, varying with species (Smith *et al*., 2000) and even among cultivars of the same species (Yanqun *et al*., 2003; Yuan *et al*., 2000). The targets of UV-B damage in the photosynthetic apparatus are the subject of current research, and evidence points to the acceptor and donor sides of photosystem II (Rodrigues *et al.,* 2006; Rensen *et al*., 2007).

Epidermal cuticle structures are capable of scattering a fraction of the incoming UV radiation (Grant *et al*., 2003). Reflectance increases in cuticles bearing dense and uniform sub-micron deposits. *P. brachyceras* cuticule configuration seems to have the requirements for a more efficient UV-scattering structure (Fig 4a). However, cuticle structures are capable to reflect UV incoming radiation to a maximum extent of 0.1. The absorbance of cuticular layers contributes much more than reflectance to UV protection, and cuticular transmittance may reach 0.5 in some individuals (Krauss *et al*., 1997). Cuticular UV transmittance is broadly variable among plants, and seems to decrease in woody and evergreen species. *In vitro*, wax solutions of *Psychotria* species did not differ at 300 nm transmittance, and *P. carthagenensis* showed a lower 280 nm transmittance. The 280 nm wavelength is poorly represented both in solar and in UVB-313EL lamp spectra (http://www.q-lab.com/EN_Weblit/QUV-LU-8160_web.pdf). Hence, this difference in wax optical properties has probably little significance to UV-tolerance

differences between *Psychotria* species. The combined analyses of the transmittance data at 300 nm and wax concentration in leaves (Fig. 4c) of *Psychotria* species suggest that, since the two species have comparable wax UV absorbance and *P. carthagenensis* has twice as much leaf wax concentration than *P. brachyceras*, the former species should be more UV-tolerant. Because the observed UV-tolerance phenotype was the exact opposite, participation of cuticular waxes in UV protection seems to be of lesser importance, and other UV-tolerance mechanisms may be involved.

Leaf tickness and tissue organization may also contribute to the UV-tolerant phenotype. Since UV-B must penetrate into the leaf to cause damage, thicker leaves would be able to shield inner layers of cells. Although average leaf thickness was similar between the tolerant and sensitive species of *Psychotria*, the presence of a chrorophyll parenchyma layer in the abaxial portion of *P. brachyceras*, separated from the adaxial palisade layer by spongy parenchyma lacking developed chloroplasts, may contribute to shielding photosynthetic tissue from UV. However, fiber-optic microprobe studies with shade and sun leaves of four evergreen sclerophylls from the Mediterranean region showed that, irrespective of leaf thickness, epidermal cells were sufficiently effective in blocking UV-B, so that only a minor fraction of it entered the mesophyll (Liakoura *et al*., 2003).

Among flavonoid compounds, two groups, flavones and flavonols, are the most active in UV protection (Julkunen-Tiito *et al*., 2005). No differences in total flavonoid contents were detected between treated and untreated leaves of both *Psychotria* species. Interestingly, *P. carthagenensis* extracts had higher flavonoid content compared to those of *P. brachyceras*. However, changes in flavonoid contents may be occurring by the

induction of a discrete individual compound, as reported for birch (Lavola, 1998). Since only flavones and flavonols form stable complexes with aluminum (Chang *et al*., 2002), a complementary method was needed to evaluate changes in anthocyanins.

Anthocyanins comprise a water-soluble group of flavonoids reportedly related to UV-B stress (Steyn *et al.,*2002). The content of total anthocyanins showed a clear induction in *P. brachyceras* leaves upon UV exposure; however, a major role for this increase in the observed UV tolerance is unlikely, since *P. carthagenensis* basal amounts of anthocyanins were approximately twice those of *P. brachyceras* induced concentrations. Kytridis and Manetas (2006) evaluated species which accumulate anthocyanins in the leaf, either in the epidermis or in the mesophyll. The increased protection against oxidative damage observed in leaves bearing mesophyll-stored anthocyanins pointed to an antioxidant rather than sunscreen role of leaf anthocyanins. The main tissue for anthocyanin accumulation is not clear from the observation of leaf sections of *Psychotria* species, but the lack of correlation between anthocyanin content and UV-tolerance is evident.

Taking into account the leaf disk weight, the doses of alkaloid applied to disks of *P. carthagenensis* treated with 1x and 10x stock solutions simulated a brachycerine content of 0.1 and 1% of the dry weight, respectively. Brachycerine contents in leaves are highly variable, ranging from a basal content from 0.02% to 0.1% and reaching up to 0.74% dry weight, depending on both genetic and environmental factors, such as season, rain regimen, light and wounding (Kerber *et al*., 2001; Gregianini *et al*., 2004). Then, in the assays of alkaloid application, a frequently found brachycerine content in leaves (0.1%

DW) was used as a basal level. An UV-induced level of 1% was used based on responses observed to UV-C (Gregianini *et al*., 2003) and UV-B stresses (Fig. 2).

Brachycerine application on *P. carthagenensis* leaves alleviated UV-driven chlorophyll decrease. The decrease in total chlorophyll concentration of control disks was not as expressive as seen in cutting samples despite the higher UV dose. This is probably because of the close contact of leaf tissues with distilled water in the leaf disk assay system, therefore keeping leaf cells constantly hydrated and, in consequence, enhancing the activity of oxidative stress defense enzymes.

The HPLC profile of brachycerine-treated *P. carthagenensis* disks revealed a weaker alkaloid signal and a new peak with slightly higher retention time in UV-exposed samples compared to non-exposed treated disks (Fig. 6 C and D). The incidence of highly energetic UV-B on the sample may have consumed brachycerine, which would be acting as a quencher of reactive oxygen species, yielding a putative degradation product. Evidence of *in vitro* chemical quenching of singlet oxygen by brachycerine has been reported (Gregianini *et al*., 2003).

A strategy used by plants in protection from UV irradiance is to store UV-absorbing compounds in the leaf epidermis, thus filtering out the harmful wavelengths (Frohnmeyer and Staiger, 2003). This function is commonly performed by phenolic compounds (Landry *et al.,* 1995; Bidel *et al.,* 2007). The putative pathway for brachycerine biosynthesis (Gregianini *et al.*, 2004) is very similar to that of strictosidine, which accumulates in epidermal cells (Murata *et al.,* 2008) and is one of the most UV-regulated alkaloids in *C. roseus* (Ouwerkerk *et al*., 1999).

Besides sunscreening, UV-protection can be provided by antioxidant compounds. Initial spin trapping studies suggested that the first reactive oxygen species generated in UV-B exposed isolated thylakoid membranes is hydroxyl radical, and the data contrasted with subsequent *in vivo* experiments, which revealed superoxide radical as the main active oxygen derivative (Hideg *et al*., 2002). Recent data supports superoxide anion as the main oxygen radical generated under UV stress, with hydroxyl arising from it via hydrogen peroxide (Šnyrychová *et al*., 2007). Brachycerine improved survival of mutant yeast strains defective in catalase and superoxide dismutase genes exposed to oxidative stress and showed scavenger properties towards hydroxyl (Nascimento *et al*., 2007) and superoxide radical (Fig. 7).

Brachycerine may act as UV-screen, preventing UV-energy absorption by the photosynthetic apparatus and its degradation, as an antioxidant, detoxifying hydroxyl and superoxide radicals, or by combining both roles. In order to elucidate this mechanism, epidermal strips of young leaves were examined for the presence of brachycerine. A significant concentration of the alkaloid was found in the epidermis, which is in agreement with the major role of this tissue in monoterpene indole alkaloid biosynthesis in *Catharanthus roseus* (Murata *et al*., 2008). Brachycerine present in mesophyll cells, in close vicinity to the photosynthetic apparatus, could play an antioxidant role, as shown for mesophyll-stored anthocyanins (Kytridis and Manetas, 2006). The epidermal fraction of brachycerine could play a sunscreen role, although an antioxidant action for epidermal brachycerine should not be discarded (Pasquali *et al*., 2006).

The two *Psychotria* species studied showed diverse responses to UV stress. None of the species had changes in total flavonoid contents in leaves upon UV stress, but anthocyanin differed in respect to basal amounts and accumulation dynamics. However, anthocyanin content did not explain the higher UV tolerance found in *P. brachyceras*. Optical properties and content of cuticular waxes did not correlate with the differences in UV-tolerance phenotype observed between the species. A major role played by the UV-induced monoterpene indole alkaloid brachycerine was postulated in the process. Data from *in vivo* "gain-of-function" experiments are in agreement with a UV-screening and/or antioxidant function of brachycerine in *P. brachyceras.* The presence of brachycerine in the epidermis at concentrations equivalent to those of whole leaf, at least in younger leaves, suggests an important role of this tissue in alkaloid metabolism. It will be interesting to investigate the alkaloid distribution in tissues of leaves of different ages and under a variety of environmental stresses, in association with transcriptome and proteome profiles.

ACKNOWLEDGEMENTS – This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico - Brazil [grant number 304374/2007-5 to AGFN]. We thank Dr. R. C. Fadanelli (Instituto de Física, UFRGS) for assistance in UV dose calculations, Ricardo J. Stein (Centro de Biotecnologia, UFRGS) for experimental suggestions, Dr. Jorge E. A. Mariath (Departamento de Botânica, UFRGS) for help with photodocumentation of histological sections, Dr. José Cláudio Fonseca Moreira (Departamento de Bioquímica, UFRGS) for the gift of Trolox sample, and Dr. Vitor A. Kerber (Departamento de Farmácia, Universidade Federal do Paraná) for contributions

on brachycerine isolation technique.

REFERENCES

Bidel LPR, Meyer S, Goulas Y, Cadot Y, Cerovic ZG. 2007. Responses of epidermal phenolic compounds to light acclimation: In vivo qualitative and quantitative assessment using chlorophyll fluorescence excitation spectra in leaves of three woody species. *Journal of Photochemistry and Photobiology B: Biology* **88,** 163-179.

Brosché M, Strid A. 2003. Molecular events following perception of ultraviolet-B radiation by plants. *Physiologia Plantarum* **117**, 1-10.

Caldwell MM. 1971. Solar UV irradiation and growth and development of higher plants. In: Giese AC ed. *Photophysiology*, Vol. 4, New York, NY:Academic Press, 131-177.

Chang C, Yang M, Wen H, Chern J. 2002. Estimation of total flavonoid contents in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis* **10,** 178-182.

Chatterjee M, Sharma P, Khurana JP. 2006. Chryptochrome 1 from *Brassica napus* is up-regulated by blue light and controls hypocotyl/stem growth and anthocyanin accumulation. *Plant Physiology* **141**, 61-74.

De Luca V, Laflamme P. 2001. The expanding universe of alkaloid biosynthesis. *Current Opinion in Plant Biology* **4,** 225-233.

Dixon R, Paiva NL. 1995. Stress-induced phenylpropanoid metabolism. *Plant Cell* **7,** 1085-1097.

Frohnmeyer H, Staiger D. 2003. Ultraviolet-B radiation-mediated responses in plants: balancing damage and protection. *Plant Physiology* **133,** 1420-1428.

Gatehouse JA. 2002. Plant resistance towards insect herbivores: a dynamic interaction. *New Phytologist* **156,** 145-169.

Grant RH, Heisler GM, Gao W, Jenks M. 2003. Ultraviolet leaf reflectance of common urban trees and the prediction of reflectance from leaf surface characteristics. *Agricultural and Forest Metereology* **120,** 127-139.

Gregianini TS, Silveira VC, Porto DD, Kerber VA, Henriques AT, Fett-Neto AG. 2003. The alkaloid brachycerine is induced by ultraviolet radiation and is a singlet oxygen quencher. *Photochemistry and Photobiology* **78**, 470-474.

Gregianini TS, Porto DD, Nascimento NC, Fett JP, Henriques AT, Fett-Neto AG. **2004.** Environmental and ontogenetic control of accumulation of brachycerine, a bioactive indole alkaloid from *Psychotria brachyceras*. *Journal of Chemical Ecology***, 30,** 2023-2036.

Harborne JB, Williams CA. 2000. Advances in flavonoid research since 1992. *Phytochemistry* **55,** 481-504.

Hideg É, Barta C, Kálai T, Vass I, Hideg K, Asada K. 2002. Detection of singlet oxygen and superoxide with fluorescent sensors in leaves under stress by photoinhibition of UV radiation. *Plant Cell Physiology* **43,** 1154-1164.

Hollósy F. 2002. Effects of ultraviolet radiation on plant cells. *Micron* **33,** 179-197.

Jansen MAK, Gaba V, Greenberg B. 1998. Higher plants and UV-B radiation: balancing damage, repair and acclimation. *Trends in Plant Science* **3,** 131-135.

Julkunen-Tiito R, Häggman H, Aphalo PJ, Lavola A, Tegelberg R, Veteli T. 2005. Growth and defense in deciduous trees and shrubs under UV-B. *Environmental Pollution* **137,** 404-414.

Kerber VA, Gregianini TS, Paranhos J, Schwambach J, Farias F, Fett JP, Fett-Neto AG, Zuanazzi JAS, Quirion J, Elisabetsky E, Henriques AT. 2001. Brachycerine, a novel monoterpene indole alkaloid from *Psychotria brachyceras*. *Journal of Natural Products* **64,** 677-679.

Krauss P, Markstädter C, Riederer M. 1997. Attenuation of UV radiation by plant cuticles from woody species. *Plant, Cell and Environment* **20,** 1079-1085.

Kytridis V, Manetas Y. 2006. Mesophyll versus epidermal anthocyanins as potential *in vivo* antioxidants: evidence linking the putative antioxidant role to the proximity of

oxy-radical source. *Journal of Experimental Botany* **57,** 2203-2210.

Landry LG, Chapple CCS, Last RL. 1995. *Arabidopsis* mutants lacking phenolic sunscreens exhibit enhanced ultraviolet-B injury and oxidative damage. *Plant Physiology* **109,** 1159-1166.

Lavola A. 1998. Accumulation of flavonoids and related compounds in birch induced by UV-B irradiance. *Tree Physiology* **18,** 53-58.

Leal MB, Elisabesky E. 1996. Absence of alkaloids in *Psychotria carthagenensis* Jacq (Rubiaceae). *Journal of Ethnopharmacology* **54**, 37-40.

Liakoura V, Bornman JF, Karabourniotis G. 2003. The ability of abaxial and adaxial epidermis of sun and shade leaves to attenuate UV-A and UV-B radiation in relation to the UV absorbing capacity of the whole leaf methanolic extracts. *Physiologia Plantarum* **117**, 33-43.

Manetas Y. 2006. Why some leaves are anthocyanic and why most anthocyanic leaves are red? *Flora* **201,** 163-177.

Mori M, Kumi Y, Ishigaki Y, Matsunaga T, Nikaido O, Kameda K, Kondo T. 2005. UV-B protective effect of a polyacylated anthocyanin, HBA, in flower petals of the

blue morning glory, *Ipomoea tricolor* cv. Heavenly Blue. *Bioorganic & Medicinal Chemistry* **13,** 2015-2020.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 709-714.

Murata J, Roepke J, Gordon H, De Luca V. 2008. The leaf epidermome of *Catharanthus roseus* reveals its biochemical specialization. *Plant Cell* **20,** 524-542.

Nascimento NC, Fragoso V, Mora DJ, Romano e Silva AC, Fett-Neto, AG, Saffi J. 2007. Antioxidant and antimutagenic effects of the crude foliar extract and the alkaloid brachycerine of *Psychotria brachyceras. Environmental and Molecular Mutagenesis* **48**, 728-734.

Ouwerkerk PBF, Hallard D, Verpoorte R, Memelink J. 1999. Identification of UV-B light responsive regions in the promoter of the tryptophan decarboxylase gene from *Catharanthus roseus*. *Plant Molecular Biology* **41,** 491-503.

Pasquali GP, Porto DD, Fett-Neto AG. 2006. Metabolic engineering of cell cultures versus whole plant complexity in production of bioactive monoterpene alkaloids: recent progress related to an old dilemma. *Journal of Bioscience and Bioengineering* **101**, 287-296.

Pilon JJ, Lambers H, Baas W, Tosserams M, Rozema J, Atkin OK. 1999. Leaf waxes of slow-growing alpine and fast-growing lowland *Poa* species: inherent differences and responses to UV-B radiation. *Phytochemistry* **50,** 571-580.

Ramani S, Shayabaskaran, C. 2008. Enhanced catharanthine and vindoline production in suspension cultures of *Catharanthus roseus* by ultraviolet-B light. *Journal of Molecular Signaling* **3,** 9-14.

Rensen JJSV, Vredenberg WJ, Rodrigues GC. 2007. Time sequence of the damage to acceptor and donor sides of photosystem II by UV-B radiation as evaluated by chlorophyll *a* fluorescence. *Photosynthesis Research* **94,** 291-297.

Rodrigues GC, Jansen MAK, Van der Noort ME, Van Rensen JJS. 2006. Evidence for the semireduced primary quinone electron acceptor of photosystem II being a photosensitizer for UV-B damage to the photosynthetic apparatus. *Plant Science* **170,** 283-290.

Ross CW. 1974. *Plant Physiology Laboratory Manual.* Wadsworth Publishing Company, Colorado State University, Belmont, CA.

Siciliano T, De Leo M, Bader A, De Tommasi N, Vrieling K, Braça A, Morelli I. 2005. Pyrrolizidine alkaloids from *Anchusta strigosa* and their antifeedant activity. *Phytochemistry* **66,** 1593-1600.

Smith J, Burrit DJ, Bannister P. 2000. Shoot dry weight, chlorophylls and UV-B-absorbing compounds as indicators of a plant sensitivity to UV-B radiation. *Annals of Botany* **86,** 1057-1063.

Šnyrychová I, Kós PB, Hideg É. 2007. Hydroxyl radicals are not the protagonists of UV-B induced damage in isolated thylakoid membranes. *Functional Plant Biology* **34,** 1112-1121.

Sprtová M, Marek MV, Nedbal L, Prášil O, Kalina J. 1999. Seasonal changes of photosynthetic assimilation of Norway spruce under the impact of UV-B radiation. *Plant Science* **142,** 37-45.

Steyn WJ, Wand SJE, Holcroft DM, Jacobs G. 2002. Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. *New Phytologist* **155,** 349-361.

Wittstock U, Gershenzon J. 2002. Constitutive plant toxins and their role against herbivores and pathogens. *Current Opinion in Plant Biology* **5,** 300-307.

Yuan L, Yanqun Z, Jianjun C, Haiyan C, Jilong Y, Zhide H. 2000. Intraspecific differences in physiological response of 20 wheat cultivars to enhanced UV-B radiation under field conditions. *Environmental and Experimental Botany* **44,** 95-103.

Yanqun Z, Yuan L, Haiyan C, Jianjun C. 2003. Intraspecific differences in physiological response of 20 soybean cultivars to enhanced UV-B radiation under field conditions. *Environmental and Experimental Botany* **50,** 87-97.

Zhishen J, Mengcheng T, Jianming W. 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry* **64,** 555-559.

Figure legends:

Fig. 1 Structure of brachycerine, a leaf alkaloid from *Psychotria brachyceras*.

Fig. 2 Brachycerine content in leaves of cuttings exposed to UV-B radiation (black bars) and control cuttings (gray bars). Data are means and standard error from one experiment with four replicates. Means sharing same letter are not significantly different at $P \leq 0.05$ by a Tukey test.

Fig. 3 Effect of UV-B exposure on chlorophyll content of two *Psychotria* species. (a), total chlorophyll content in leaves of *P. brachyceras* cuttings; (b), chlorophyll *a/b* ratio in leaves of *P. brachyceras*; (c), total chlorophyll content in leaves of *P. carthagenensis*; (d), chlorophyll *a/b* ratio in leaves of *P. carthagenensis.* Data of total chlorophylls are expressed on an extracted dry weight (DW) basis. Data are means and standard error

from three experiments. Means sharing same letter are not significantly different at $P \leq 0.05$ by a Tukey test.

Fig. 4 Cuticular wax characterization of *Psychotria* species. (a) and (b), *P. brachyceras* (a) and *P. carthagenensis* (b) leaf sections stained with Sudan III. Arrowhead and arrows indicate predominantly uniform layer of *P. brachyceras* and the presence globular structures in *P. carthagenensis*, respectively.(c), leaf cuticular wax content of the *Psychotria* species. The asterisk indicates statistical difference of means by *t* test for $P \leq 0.05$.

Fig. 5 Effect of UV-B exposure (UV) or white light only (WL) on total flavonoids (a) and total anthocyanins (b) of two *Psychotria* species. Total flavonoids are expressed in quercetin equivalents (QE) per extracted dry weight (DW), and anthocyanins by optical density (OD) per extracted dry weight (DW). Data are means and standard error from three experiments. Means sharing same letter are not significantly different at $P \leq 0.05$ by a Tukey test.

Fig. 6 In vivo UV protection assay. (a) and (b): *P. carthagenensis* leaf disks were treated with brachycerine solutions of either 1 g/l (Brach 1x) or 10 g/l (Brach 10x) concentrations. Control disks were treated with solvent only (MeOH). Samples were then exposed to white light only (WL) or white light plus UV radiation (UV) for 4 days.

Total chlorophylls (a) and chlorophyll *a/b* ratios (b) are represented. Data are means and standard error of two experiments. Means sharing a letter are not significantly different at $P \leq 0.05$ by a Tukey test. (c) and (d): HPLC profiles of treated disks (10x stock solution) when exposed to white light only (c) or white light plus UV radiation treatments (d). The arrow indicates the brachycerine peak.

Fig. 7 Superoxide scavenging assay. The in vitro test is based on the prevention of nitrobluetetrazolium (NBT) oxidation by riboflavin-generated superoxide anions upon illumination with white light. Trolox is a powerful superoxide quencher used as positive control. Results are data from a representative experiment done in triplicate. The different letters above mean and standard error values indicate statistical difference by Tukey test for $P \leq 0.05$.

Figure 1

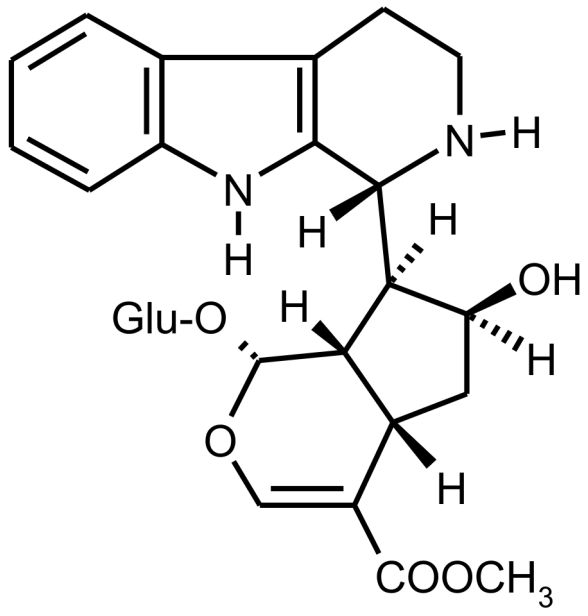


Figure 2

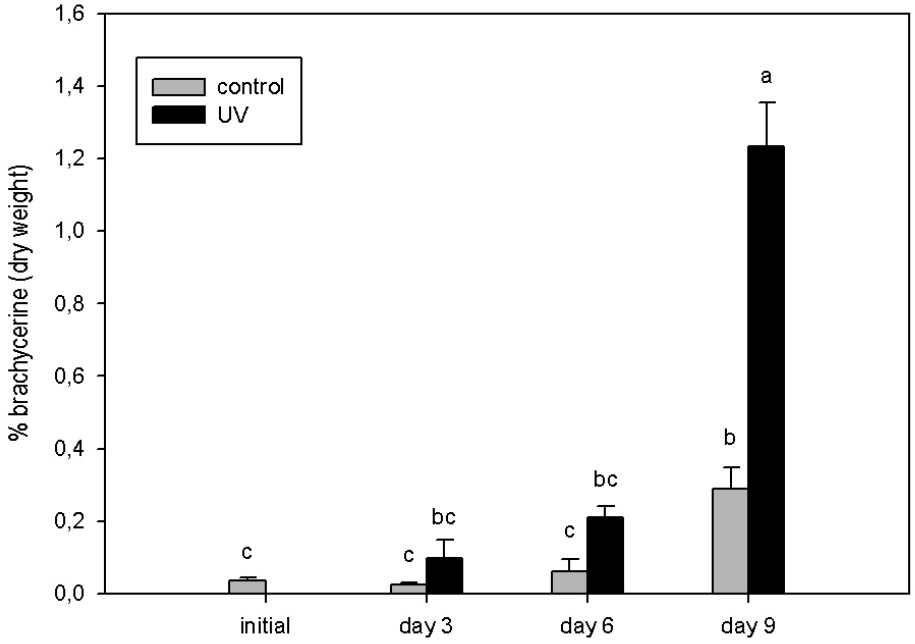

1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
% brachycerine (dry weight)
control
UV
c
c
bc
c
bc
b
a
initial
day 3
day 6
day 9

Figure 3

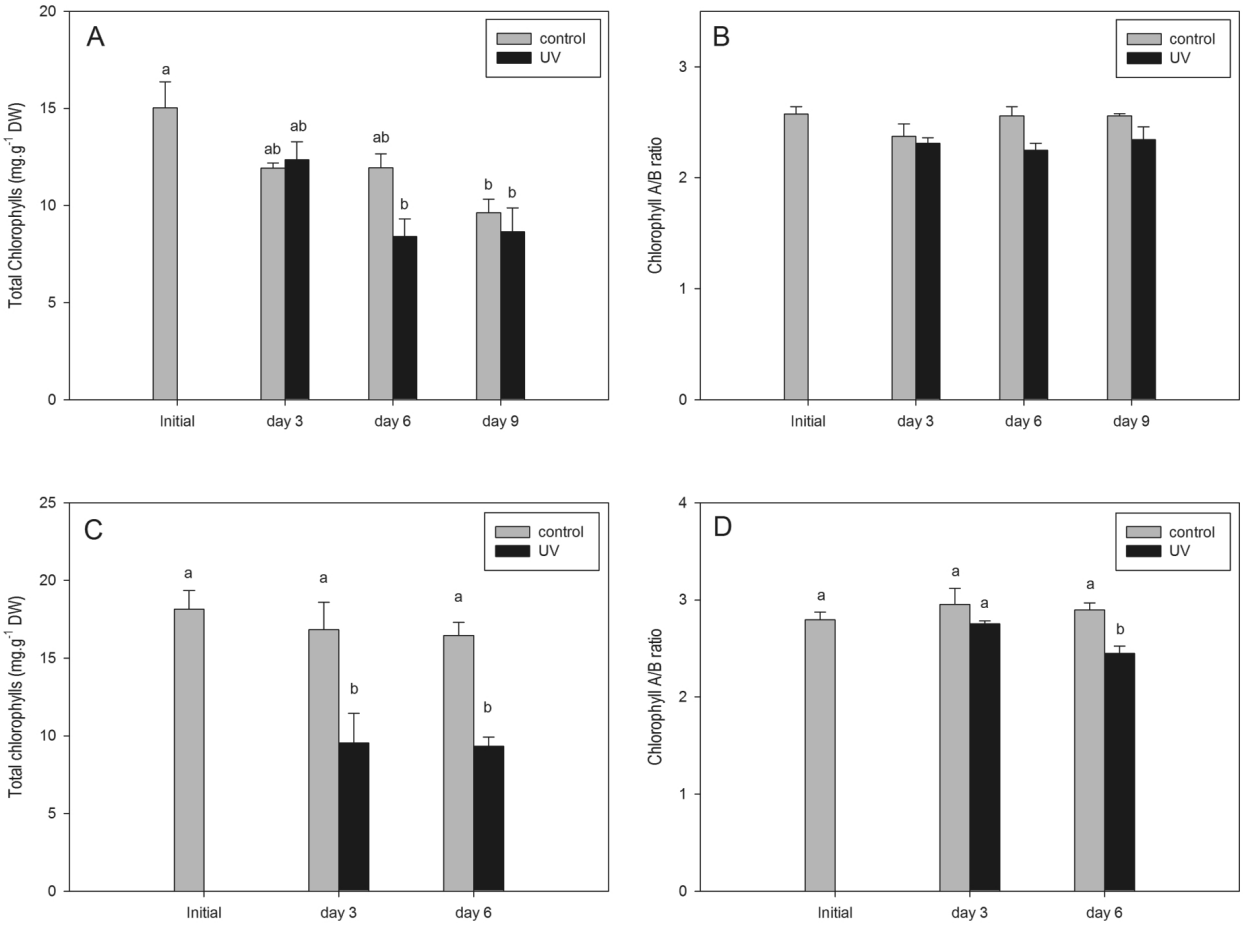

A
control
UV
Total Chlorophylls (mg.g-1 DW)
20
15
10
5
0
a
ab
ab
ab
b
b
b
Initial
day 3
day 6
day 9
B
control
UV
Chlorophyll A/B ratio
3
2
1
0
Initial
day 3
day 6
day 9
C
control
UV
Total chlorophylls (mg.g-1 DW)
25
20
15
10
5
0
a
a
b
a
b
Initial
day 3
day 6
D
control
UV
Chlorophyll A/B ratio
4
3
2
1
0
a
a
a
a
b
Initial
day 3
day 6

Figure 4

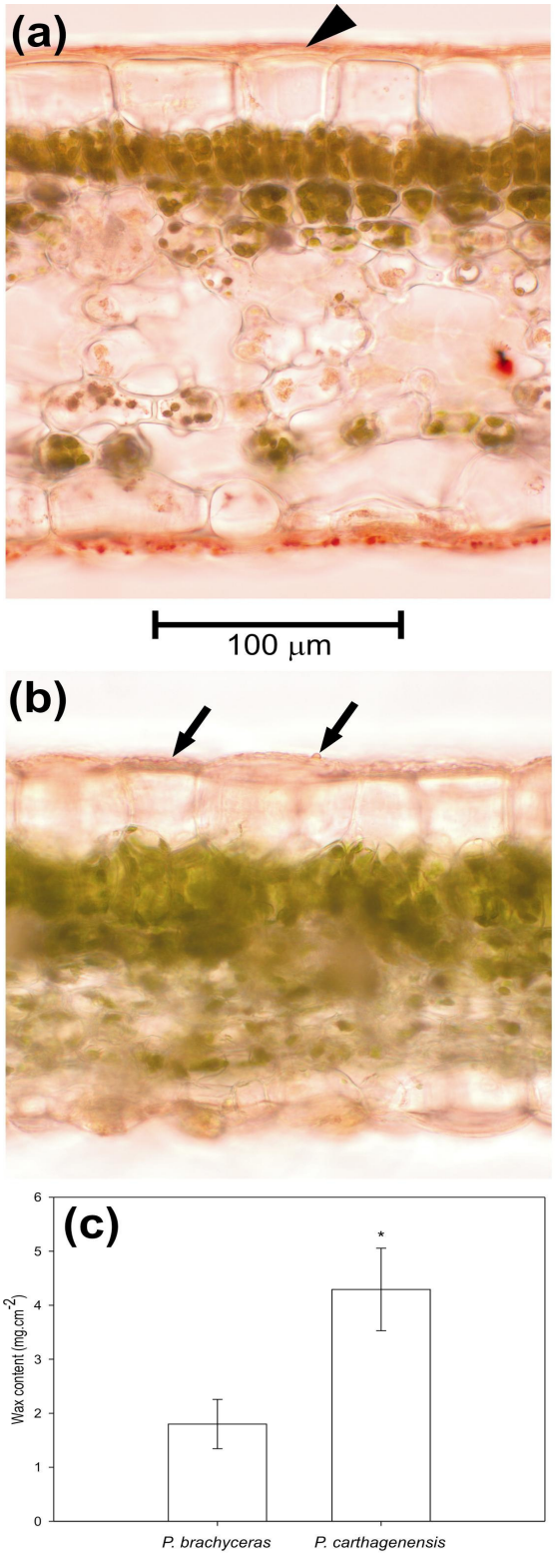

(a)
100 µm
(b)
(c)
Wax content (mg.cm-2)
0
1
2
3
4
5
6
*
P. brachyceras
P. carthagenensis

Figure 5

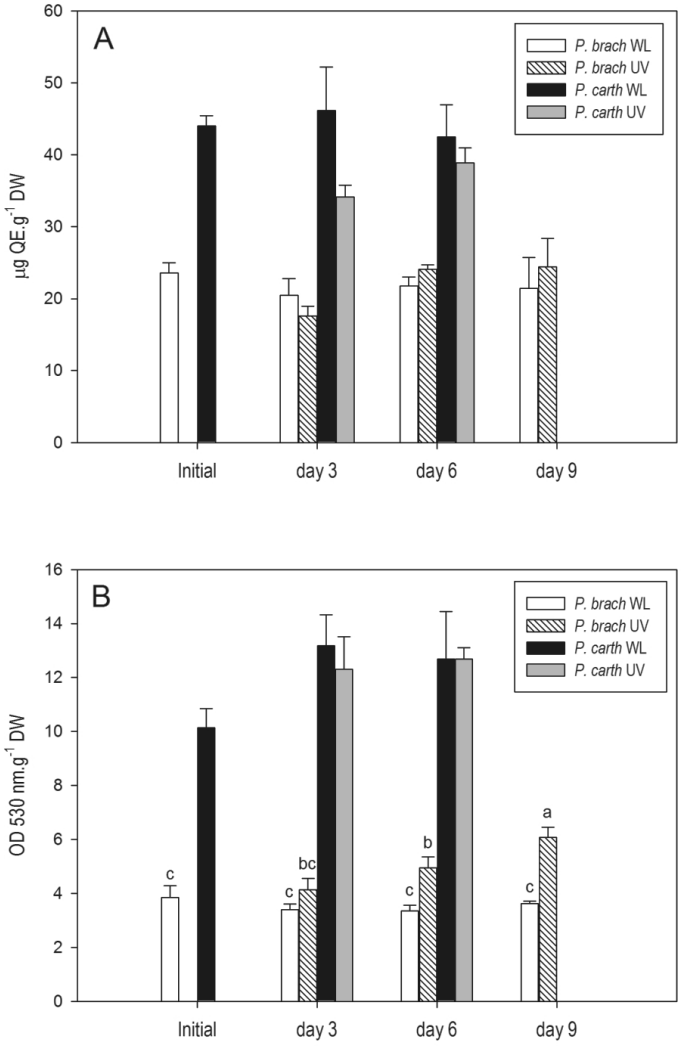
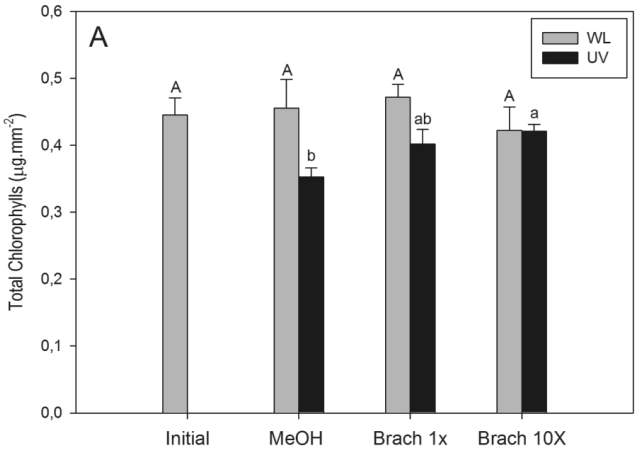

A
P. brach WL
P. brach UV
P. carth WL
P. carth UV
µg QE.g-1 DW
0
10
20
30
40
50
60
Initial
day 3
day 6
day 9
B
P. brach WL
P. brach UV
P. carth WL
P. carth UV
OD 530 nm.g-1 DW
0
2
4
6
8
10
12
14
16
c
c
bc
c
b
c
a
Initial
day 3
day 6
day 9

Figure 6


A
WL
UV
Total Chlorophylls (µg.mm-2)
0,6
0,5
0,4
0,3
0,2
0,1
0,0
A
A
b
A
ab
A
a
Initial
MeOH
Brach 1x
Brach 10X

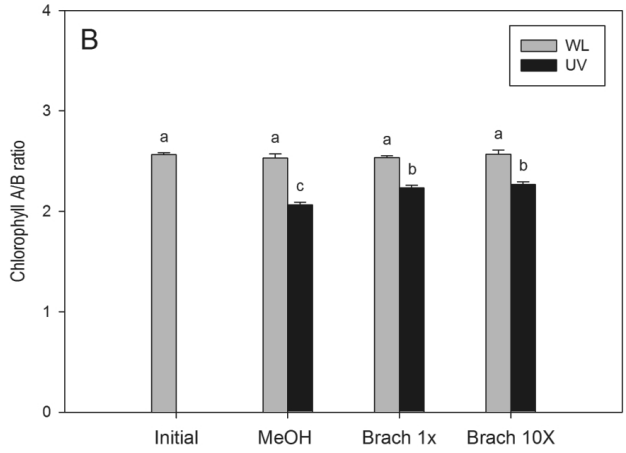
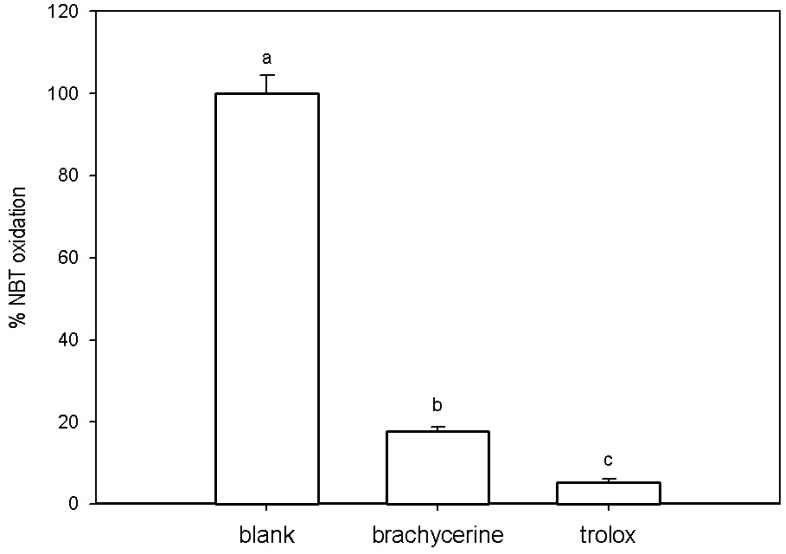

B
WL
UV
Chlorophyll A/B ratio
4
3
2
1
0
a
a
c
a
b
a
b
Initial
MeOH
Brach 1x
Brach 10X

Figure 7


120
100
80
60
40
20
0
% NBT oxidation
a
b
c
blank
brachycerine
trolox

6. DISCUSSÃO GERAL

6.1. DANO MECÂNICO E HERBIVORIA

Uma série de experimentos com amostras de *Psychotria brachyceras* foi realizada em trabalhos anteriores (Gregianini *et al.,* 2003a; Gregianini, 2003b; Gregianini *et al.,* 2004) testando o efeito de uma variedade de estresses e elicitores sobre o acúmulo de braquicerina. Alguns desses tratamentos induziram acúmulos significativos do alcalóide, como o dano mecânico, a aplicação de jasmonato e a exposição à ultravioleta. Estacas de *P. brachyceras* que sofrem dano mecânico nas folhas apresentam acúmulo de braquicerina estatisticamente maior no segundo dia após o dano em folhas danificadas (Gregianini *et al.,* 2004). Com o objetivo de melhor caracterizar esse fenômeno, foram feitos novos experimentos com coletas visando melhor resolução da curva de resposta temporal de braquicerina. A indução da concentração do alcalóide tornou-se perceptível a partir de 24 horas após o dano (manuscrito 1, Fig. 1a).

A indução de respostas é uma estratégia que tem várias vantagens em relação ao acúmulo constitutivo (não-regulado) de compostos de defesa (Agrawal & Karban, 1999). A hipótese de que a produção de defesas consome recursos importantes para o desenvolvimento e a reprodução tem sido apoiada por dados recentes. Variedades selvagens de tabaco (*Nicotiana attenuata*) produzem inibidores de protease como parte de suas defesas contra herbívoros, com destaque para a larva do especialista *Manduca sexta*. Plantas silenciadas na produção de inibidores de protease cresceram mais, tiveram florescimento antecipado e produziram mais frutos (Zavala *et al.,* 2004). Em outro estudo, duas espécies de *Lupinus* sp. produtoras de alcalóides quinolizidínicos tóxicos, *L. albus* e *L. angustifolius*, foram testadas quanto ao crescimento e desempenho reprodutivo após dano mecânico. *L. albus* aumenta o conteúdo foliar de alcalóides quando sofre dano mecânico, enquanto que plantas de *L. angustifolius* não mudam a concentração de alcalóides sob esse tratamento. Plantas de *L. angustifolius* foram capazes de compensar perdas de biomassa, ao contrário de plantas de *L. albus*, que tiveram seu desempenho reprodutivo comprometido (Vilariño & Ravetta, 2008). Os experimentos com *Nicotiana attenuata* e com as espécies de *Lupinus* confirmam a hipótese do custo metabólico na produção de defesas. Seguindo essa linha de raciocínio, a indução do acúmulo de braquicerina por

dano mecânico, aplicação de jasmonato e radiação ultravioleta sugere que o alcalóide desempenha uma função nessas situações.

As respostas induzidas por dano mecânico nas plantas não necessariamente se traduzem em maior resistência contra ataques de herbívoros. O próximo passo nessa avaliação é o teste da influência da resposta induzida sobre o desempenho do herbívoro (Karban & Myers, 1989). Como não há descrição na literatura de folivoria em *P. brachyceras*, nosso modelo experimental de teste da hipótese da braquicerina como defesa química direta foi constituído de herbívoros generalistas. A literatura é rica em exemplos de alcalóides que cumprem uma função protetora contra ataques de herbívoros. Além dos alcalóides quinolizidínicos de *Lupinus* sp. já citados, alcalóides pirrolizidínicos de *Anchusta strigosa* (Siciliano *et al.,* 2005), alcalóides tropânicos de *Atropa acuminata* (Khan & Harborne, 1991) e gramina, um alcalóide indólico de cevada (Velozo et al., 1999) já tiveram descrições de ação repelente de herbívoros. O alcalóide mais bem estudado na interação com herbívoros é a nicotina. Sua ação de proteção já é bem estabelecida (Baldwin, 1988). O efeito da nicotina no sistema nervoso é igualmente bem conhecido – sabe-se que o alcalóide age como agonista do receptor de acetilcolina nicotínico da membrana pós-sináptica (Tomizawa & Casida, 2002).

No entanto, contrariando a hipótese inicial, os dois bioensaios realizados não demonstraram ação tóxica de braquicerina em todas as concentrações testadas. Extratos de *P. carthagenensis*, nos quais não foram detectados alcalóides (Leal & Elisabetsky, 1996), foram mais tóxicos do que os de *P. brachyceras* no ensaio com moluscos. Análises posteriores revelaram a presença de taninos nos tecidos de *P. carthagenensis* e não nos de *P. brachyceras*, o que poderia explicar esse resultado. Os dados apontam para o exato oposto do inicialmente pensado – *P. carthagenensis*, que não acumula alcalóides e aparece freqüentemente predada a campo, revelou-se mais tóxica do que *P. brachyceras*, cujas plantas crescem praticamente intactas em seu ambiente natural.

Essa última observação fez surgir uma nova hipótese do papel de braquicerina em resposta a dano mecânico. Indivíduos de *P. brachyceras* parecem sofrer danos apenas esporádicos, ao contrário de *P. carthagenensis* e outras espécies vegetais que aparentam ser sistematicamente predadas. Outra observação que distingue braquicerina em relação a alcalóides tóxicos mais estudados é a localização do acúmulo induzido. A concentração de braquicerina induzida por dano foliar aumenta apenas na folha que sofreu o dano

(Gregianini *et al.,* 2004) e não de forma sistêmica, como ocorre com vários alcalóides tóxicos estudados (Baldwin *et al*., 1997; Velozo *et al*., 1999; Vilariño *et al.,* 2005). Esse dado reforça a idéia de que braquicerina tem uma ação relacionada ao ferimento em si, e não a alguma interação ecoquímica.

As plantas podem tolerar em média 18% de perda de área foliar sem conseqüências significativas para seu crescimento e reprodução (Strauss & Agrawal, 1999). Essa capacidade das plantas de tolerar o dano a seus tecidos é considerada uma defesa vegetal tanto quanto a resistência por compostos químicos tóxicos (Strauss & Agrawal, 1999; Stowe *et al.,* 2000). Em um recente estudo, Agrawal e Fishbein (2008) construíram uma filogenia molecular de 36 espécies de *Asclepias* sp. e duas da aparentada *Gomphocarpus* sp., relacionando a diversificação de espécies e a presença de defesas diretas (resistência) como compostos tóxicos, látex e tricomas. Os autores observaram um declínio filético na presença de resistência e uma maior predominância de rebrote, um mecanismo de tolerância, em espécies mais derivadas. Além do rebrote, mecanismos de tolerância incluem alocação de recursos para órgãos subterrâneos e aumento na taxa de crescimento e fotossíntese (Strauss & Agrawal, 1999; Stowe *et al.,* 2000). Esses mecanismos descrevem a resposta da planta como um todo, sem especificar o comportamento metabólico dos tecidos próximos ao dano em plantas tolerantes.

O dano mecânico causa uma explosão oxidativa no tecido foliar danificado (Orozco-Cardenas & Ryan, 1999). A produção de peróxido de hidrogênio sinaliza a presença do dano mecânico de forma sistêmica (Sagi *et al.,* 2004), mas o excesso deste radical pode danificar componentes celulares e levar à morte celular programada (Van Breusegem *et al.,* 2001; Gechev & Hille, 2005). O peróxido de hidrogênio parece ser derivado do ânion superóxido via NADPH oxidases (Orozco-Cardenas & Ryan, 1999; Apel & Hirt, 2004). Mecanismos de detoxificação de peróxido de hidrogênio, assim como outros radicais ativos de oxigênio, são compostos de sistemas enzimáticos e não-enzimáticos. Os sistemas não-enzimáticos incluem os bem estudados ascorbato e glutationa, mas já foram descritos como participantes desse sistema moléculas do metabolismo secundário como flavonóides, carotenóides e alcalóides (Apel & Hirt, 2004).

A braquicerina possui considerável atividade antioxidante e ampla distribuição nos tecidos das partes aéreas de *P. brachyceras* (Gregianini *et al.,* 2003a; Gregianini *et al.,* 2004; Nascimento *et al.,* 2007 e Fig. 7 do manuscrito 2.). Com o intuito de investigar a

participação de braquicerina na detoxificação de radicais ativos de oxigênio, um bioteste foi utilizado no qual discos de folhas de *Coleus blumei* foram imersos em 3,3-diaminobenzidina (DAB), um composto que forma polímeros amarronzados insolúveis na presença de peróxido de hidrogênio (Thordal-Christensen *et al.,* 1997). A presença de braquicerina inibiu parcialmente, mas de forma visível, a coloração por DAB, o que sugere que o alcalóide possa estar contribuindo para a preservação dos tecidos em eventos de estresse oxidativo induzidos por dano mecânico.

A presença de compostos secundários antioxidantes pode proteger os tecidos do estresse oxidativo gerado por ferimento. Folhas com alto teor de antocianinas e folhas desprovidas do pigmento foram danificadas, e o acúmulo de H_2O_2 foi monitorado (Gould et al., 2002). A presença de antocianinas nas folhas fez com que o H_2O_2 gerado por ferimento fosse detoxificado com mais rapidez.

A braquicerina tem presença constante nos tecidos das partes aéreas de *P. brachyceras*, o que pode contribuir para a imediata detoxificação de radicais ativos de oxigênio. No entanto, o acúmulo induzido inicia-se apenas 24 horas após o dano, e a produção de peróxido de hidrogênio provocada por dano é máxima entre 4 a 6 horas após o ferimento (Orozco-Cardenas & Ryan, 1999). O alcalóide pode ter uma função diferente nos tecidos entre 24 e 48 horas após o dano mecânico, talvez constituindo uma resposta de antecipação contra estresses futuros.

6.2. RADIAÇÃO ULTRAVIOLETA

O excesso de radiação ultravioleta (UV) causa uma variedade de danos em células vegetais (Hollósy, 2002; Frohnmeyer & Staiger, 2003). O aumento da incidência do ultravioleta-B (UV-B, 280-320 nm) na superfície terrestre em consequência de danos na camada de ozônio é um foco de grande esforço de pesquisa, já que compromete o crescimento vegetal e pode prejudicar a produção de alimentos e outros insumos. O UV-B é diretamente absorvido por componentes celulares como DNA, proteínas e fitormônios, podendo degradar essas moléculas (Hollósy, 2002; Frohnmeyer & Staiger, 2003). Além disso, a exposição ao UV-B desencadeia estresse oxidativo, provocando peroxidação de lipídios e dano às membranas, entre outros processos (Jansen *et al.,* 1998).

A irradiação por UV-B causa danos pronunciados no aparelho fotossintético. O alvo primário da radiação parece ser o fotossistema II, mais precisamente as quinonas da

cadeia transportadora de elétrons (van Rensen *et al.,* 2007). A absorção do UV-B pelos componentes do fotossistema II causa a produção do radical superóxido (Hideg *et al.,* 2002), que, por sua vez, converte-se a H_2O_2 e, em seguida, a radical hidroxila no ambiente celular (Šnyrychová *et al.,* 2007).

A exposição de discos foliares ou folhas de estacas de *P. brachyceras* ou *P. carthagenensis* a UV-B resultou em redução da concentração das clorofilas. Essa redução foi mais acentuada em amostras de *P. carthagenensis*, que não sobreviveram até o nono dia de tratamento, no caso do experimento com estacas. Outro comportamento comum entre as duas espécies foi a redução da razão entre as clorofilas *a* e *b*.

O efeito do UV-B sobre as clorofilas é bastante variável entre espécies vegetais, e não necessariamente prediz a sensibilidade de certa espécie a condições de alta intensidade de UV-B (Smith *et al.,* 2000). No entanto, o teor de clorofilas é um indicador confiável da integridade dos fotossistemas, que é o principal processo gerador de biomassa e, portanto, de crescimento. O estresse por UV-B causa decréscimo do conteúdo total e da razão *a/b* de clorofilas em soja (Yanqun *et al.,* 2003), trigo (Yuan *et al.,* 2000) e abeto (*Picea abies*, Šprtová *et al.,* 1999). A redução da razão entre clorofilas é devida ao maior dano causado pelo UV-B nos fotossistemas ricos em clorofila *a*, em especial o fotossistema II, como descrito acima.

Plantas respondem ao excesso de UV-B por meio de diversos mecanismos, do enrolamento das folhas à síntese de compostos que absorvem esses comprimentos de onda (Jansen *et al.,* 1998). Ceras epicuticulares já foram citadas como protetoras contra UV-B (Hollósy, 2002; Knust & Samuels, 2003), mas essa função não parece ser ubíqua (Pilon *et al.,* 1999). No nosso estudo, *P. carthagenensis* acumulou mais que o dobro da quantidade de ceras do que *P. brachyceras*. Mesmo assim, os bioensaios revelaram que estacas de *P. carthagenensis* são bem mais sensíveis a UV-B do que *P. brachyceras*, portanto o acúmulo de ceras não parece estar relacionado com proteção a UV-B.

Os compostos secundários mais comumente citados na defesa contra a exposição à UV-B são os compostos fenólicos. Essas moléculas são transparentes à luz visível, porém absorvem a radiação ultravioleta na faixa do UV-B, agindo como um filtro solar (Jansen *et al.,* 1998). Além disso, são potentes antioxidantes. Plantas de *Arabidopsis thaliana* com a produção de compostos fenólicos silenciada são extremamente sensíveis ao UV-B (Landry *et al.,* 1995). Dentro dos compostos fenólicos, os flavonóides são descritos como eficazes

na proteção a esse estresse (Middleton & Teramura, 1993; Li *et al.,* 1993). Dois grupos de flavonóides são os mais ativos na proteção contra UV-B: as flavonas e os flavonóis (Julkunen-Tiito *et al.,* 2005). Moléculas desses grupos possuem motivos estruturais que conferem alta atividade antioxidante (Rice-Evans *et al.,* 1997; Magnani *et al.,* 2000).

Com a finalidade de caracterizar a produção dos compostos secundários de *P. brachyceras* com relevância na proteção contra UV, os flavonóides totais de folhas de estacas da espécie expostas a UV-B foram quantificados. O teor total de flavonas e flavonóis em tecidos vegetais pode ser estimado espectrofotometricamente pela capacidade desses compostos de ligar-se ao íon alumínio (Al^{3+}), produzindo uma coloração estável (Chang *et al.,* 2002). Não foi detectada influência da exposição ao UV-B no teor total destes flavonóides em estacas de *P. brachyceras*. Como discutido no manuscrito II, as plantas podem acumular um conjunto de flavonóides composto de várias moléculas, e a regulação por ultravioleta nem sempre atinge todas elas, gerando uma influência bastante discreta em termos de indução da concentração total de flavonóides. A ausência de uma regulação perceptível do teor total de flavonóides por ultravioleta pode significar que não ocorre um investimento significativo de recursos na proteção contra esse estresse através desses compostos.

Antocianinas são flavonóides solúveis que conferem pigmentação a certos tecidos. Sua função em folhas não é totalmente conhecida, mas hipóteses sugerem participação na defesa contra radiação ultravioleta e estresse oxidativo (Manetas, 2005). Folhas que acumulam antocianinas em células do mesofilo resistem melhor a estresse por ânion superóxido do que folhas com esse pigmento acumulado na epiderme (Kytridis & Manetas, 2005). O ânion superóxido é o principal radical ativo de oxigênio gerado em estresse por UV-B (Hideg et al., 2002; Šnyrychová et al., 2007). As antocianinas podem ter uma função antioxidante em folhas expostas a essa radiação. A indução do acúmulo de antocianinas em folhas de *P. brachyceras* expostas a UV-B pode estar relacionada à necessidade de neutralizar o estresse oxidativo gerado por UV-B.

Existem poucas descrições de funções de alcalóides em relação ao excesso de ultravioleta em plantas. A própria braquicerina já foi citada como um dos poucos exemplos de alcalóide com função em relação a estresse por UV (Jansen *et al.,* 2008). O alcalóide já havia sido descrito como ativo em extiguir o oxigênio singleto (Gregianini *et al.,* 2003), radical produzido pela liberação de elétrons de clorofilas em estado tripleto (Schweitzer &

Schmidt, 2003). Além do oxigênio singleto, a braquicerina mostrou-se um forte antioxidante contra o radical superóxido (Manuscrito II, Fig. 7).

A proteção contra radiação UV-B por compostos antioxidantes já foi descrita em outros sistemas vivos. A pitiriacitrina, um alcalóide indólico, é produzida pela levedura *Malassezia furfur* e é capaz de diminuir o efeito inibitório do UV-B sobre seu crescimento (Mayser *et al.,* 2002). A N-acetilcisteína é um tiol altamente antioxidante principalmente de radicais hidroxila. Organismos fotossintetizantes cultivados na presença desse composto sofreram menor dano oxidativo quando expostos ao UV-B (Malanga *et al.,* 1999; He & Häder, 2002). A N-acetilcisteína é completamente transparente ao UV-B, portanto sua função de proteção deve-se à sua capacidade antioxidante.

A tolerância ao UV-B parece depender de mecanismos antioxidantes. Em *Arabidopsis thaliana*, o silenciamento do gene *vtc1* resulta em plantas com menores concentrações de ácido ascórbico. Essas plantas apresentam maior sensibilidade ao ultravioleta (Gao & Zhang, 2008). A estratégia alternativa à proteção por mecanismos antioxidantes é a desempenhada por flavonóides ou outros compostos fenólicos que se acumulam na epiderme e/ou na cutícula atuando como filtros solares, bloqueando a incidência de UV-B enquanto são transparentes à radiação fotossinteticamente ativa (Landry *et al.,* 1995).

A braquicerina é encontrada em células epidérmicas. Entretanto, apesar de o espectro de absorção de radiação da braquicerina incluir comprimentos de onda da faixa do UV-B (entre 280 e 300 nm; Gregianini et al., 2003a), apenas uma curta faixa de comprimentos de onda coincide com o UV-B solar que chega à superfície terrestre (290 a 315 nm; Hollósy, 2002). Essa observação, juntamente com o resultado do ensaio de atividade antioxidante *in vitro*, indica que a principal função da braquicerina na tolerância a UV-B é como antioxidante, provavelmente de radicais superóxido.

6.3. CONSIDERAÇÕES FINAIS

Os dados dos experimentos com dano mecânico e radiação ultravioleta sugerem que a braquicerina exerce um papel fundamentalmente antioxidante ou modulador de estresse oxidativo em folhas de *P. brachyceras*. O alcalóide pode estar agindo como um agente de tolerância, detoxificando os tecidos e impedindo a inutilização dos órgãos por estresses ambientais. As funções do alcalóide como antioxidante de amplo espectro

podem atuar em outras circunstâncias adversas, como desidratação e fotoinibição. Esse tipo de função não é comum para um alcalóide, o que torna o estudo da braquicerina uma interessante contribuição para o entendimento da função de alcalóides em plantas.

7. RESULTADOS PRINCIPAIS

- A indução de braquicerina por dano mecânico é restrita às folhas e surge 24 horas após a aplicação do dano;
- O alcalóide não exerce efeito tóxico ou deterrente em dois herbívoros generalistas testados;
- A presença do alcalóide em *Coleus blumei* restringe a produção de peróxido na explosão oxidativa gerada por dano mecânico, podendo ter função semelhante em folhas danificadas de *P. brachyceras;*
- A concentração foliar de braquicerina aumenta em resposta à radiação UV-B e *P. brachyceras* mostra-se muito mais tolerante a UV do que *P. carthagenensis*;
- O estresse por UV-B não causa alteração significativa na concentração de flavonóides totais nas folhas da planta;
- As antocianinas totais de *P. brachyceras* sofrem indução por exposição a UV-B, mas atingem concentração menor do que a basal presente em *P. carthagenensis*;
- A aplicação de braquicerina sobre a epiderme adaxial é capaz de proteger a integridade dos aparatos fotossintéticos de *P. carthagenensis* expostos ao UV-B;
- A braquicerina tem significativa atividade antioxidante frente ao ânion superóxido, o que pode ser o mecanismo principal de proteção exercido pelo alcalóide.

8. CONCLUSÕES

- O aumento na concentração foliar de braquicerina induzido por dano sugere uma participação do alcalóide nessa situação;
- A braquicerina não é capaz de repelir o consumo de tecidos por herbívoros generalistas;
- O alcalóide possui um papel antioxidante, podendo participar de uma estratégia de tolerância;
- O acúmulo de braquicerina é induzido por UV-B e sua indução é coordenada com a de antocianinas sob esse tratamento;
- Folhas de *P. brachyceras* são mais tolerantes a UV-B em relação a *P. carthagenensis,* mesmo tendo menores teores de flavonóides, antocianinas e ceras epicuticulares;
- A aplicação de braquicerina protege clorofilas de *P. carthagenensis* da degradação por UV-B;
- No estresse por exposição ao UV-B, o mecanismo de ação do alcalóide parece ser primariamente antioxidante em relação ao ânion superóxido.

9. REFERÊNCIAS BIBLIOGRÁFICAS

Agrawal, A. A. & Fishbein, M. 2008. Phylogenetic escalation and decline of plant defense strategies. **Proceedings of the National Academy of Sciences of the USA 105(29):** 10057-10060.

Agrawal, A. A. & Karban, R. 1997. Leaf domatia mediate plant-arthropod mutualism. **Nature 387:** 562-563.

Agrawal, A. A. & Karban, R. 1999. Why induced defenses may be favored over constitutive strategies in plants. *In*: Tollrian, R. & Harvell, C. D. (eds.) *The*

ecology and evolution of inducible defenses. Princeton University Press, Princeton, USA, p. 45-61.

Apel, K. & Hirt, H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. **Annual Review in Plant Biology 55:** 373-399.

Bakkali, F.; Averbeck, S.; Averbeck, D.; Idaomar, M. 2008. Biological effects of essential oils - a review. **Food and Chemical Toxicology 46:** 446-475.

Baldwin, I. T. 1988. Short-term damage-induced increases in tobacco alkaloids protects plants. **Oecologia 75:** 367-370.

Baldwin, I. T.; Zhang, Z.; Diab, N.; Ohnmeiss, T. E.; McCloud, E. S.; Lyndis, G. Y.; Schmelz, E. A. 1997. Quantification, correlations and manipulations of wound-induced changes in jasmonic acid and nicotine in *Nicotiana sylvestris.* **Planta 201:** 397-404.

Balunas, M. J. & Kinghorn, A. D. 2005. Drug discovery from medicinal plants. **Life Sciences 78:** 431-441.

Both, F. L.; Kerber, V. A.; Henriques, A. T.; Elisabetsky, E. 2002a. Analgesic properties of umbellatine from *Psychotria umbellata*. **Pharmaceutical Biology 40(5):** 336-341.

Both, F. L.; Farias, F. M.; Nicoláo, L. L.; Misturini, J.; Henriques, A. T.; Elisabetsky, E. 2002b. Avaliação da atividade analgésica de extratos alcaloídicos de espécies de *Psychotria*. **Revista Brasileira de Plantas Medicinais 5(1):** 41-45.

Both, F. L.; Meneghini, L.; Kerber, V. A.; Henriques, A. T.; Elisabetsky, E. 2005. Psychopharmacological profile of the alkaloid Psychollatine as a $5HT2_{A/C}$ serotonin modulator. **Journal of Natural Products 68:** 374-380.

Boudet, A. 2007. Evolution and current status of research in phenolic compounds. **Phytochemistry 68:** 2722-2735.

Bourgaud, F.; Gravot, A.; Milesi, S.; Gontier, E. 2001. Production of plant secondary metabolites: a historical perspective. **Plant Science 161:** 839-851.

Burgoyne, P. M.; van Wyk, A. E.; Anderson, J. M.; Schrire, B. D. 2005. Phanerozoic evolution of plants on the African plate. **Journal of African Earth Sciences 43:** 13-52.

Cano, A.; Alcaraz, O.; Arnao, M. B. 2003. Free-radical scavenging activity of indolic compounds in aqueous and ethanolic media. **Analytical and Bioanalytical Chemistry 376:** 33-37.

Carvalho, C. C. C. R. & Fonseca, M. M. R. 2005. Biotransformation of terpenes. **Biotechnology Advances 24:** 134-142.

Chang, C.; Yang, M.; Wen, H.; Chern, J. 2002. Estimation of total flavonoid contents in propolis by two complementary colorimetric methods. **Journal of Food and Drug Analysis 10(3):** 178-182.

Cushnie, T. P. T. & Lamb, A. J. 2005. Antimicrobial activity of flavonoids. **International Journal of Antimicrobial Agents 26:** 343-356.

De Luca, V. & Laflamme, P. 2001. The expanding universe of alkaloid biosynthesis. **Current Opinion in Plant Biology 4:** 225-233.

De Luca, V. & St-Pierre, B. 2000. The cell and developmental biology of alkaloid biosynthesis. **Trends in Plant Science 5(4):** 168-173.

Di Fiore, S.; Li, Q.; Leech, M. J.; Schuster, F.; Emans, N.; Fischer, R.; Schillberg, S. 2002. Targeting tryptophan decarboxylase to selected subcellular compartments of tobacco plants affects enzyme stability and *in vivo* function and leads to a lesion-mimic pPhenotype. **Plant Physiology 129:** 1160-1169.

Dillenburg, C. & Porto, M. L. 1985. **Boletim do Instituto de Biociências** – n° 39. UFRGS, Porto Alegre, 82 p.

Dixon, R. A. & Paiva, N. L. 1995. Stress-induced phenylpropanoid metabolism. **Plant Cell 7:** 1085-1097.

Dudareva, N.; Andersson, S.; Orlova, I.; Gatto, N.; Reichelt, M.; Rhodes, D.; Boland, W.; Gershenzon, J. 2004. The nonmevalonate pathway supports both monoterpene and sesquiterpene formation in snapdragon flowers. **Proceedings of the National Academy of Sciences of USA 102(3):** 933-938.

Elisabetsky, E.; Amador, T. A.; Albuquerque, R. R.; Nunes, D. S.; Carvalho, A. C. T. 1995. Analgesic activity of *Psychotria colorata* (Willd. Ex R. & S.) Muell. Arg. alkaloids. **Journal of Ethnopharmacology 48:** 77-83.

Facchini, P. J.; Huber-Allanach, K. L.; Tari, L. W. 2000. Plant aromatic L-amino acid decarboxylases: evolution, biochemistry, regulation and metabolic engineering applications. **Phytochemistry 54:** 121-138.

Facchini, P. & Park, S. 2003. Developmental and inducible accumulation of gene transcripts involved in alkaloid biosynthesis in opium poppy. **Phytochemistry 64:** 177-186.

Facchini, P. J. & St Pierre, B. 2005. Synthesis and trafficking of alkaloid biosynthetic enzymes. **Current Opinion in Plant Biology 8:** 657-666.

Fragoso, V.; Nascimento, N. C.; Moura, D. J.; Silva, A. C. R.; Richter, M. F.; Saffi, J.; Fett-Neto, A. G. 2008. Antioxidant and antimutagenic properties of the monoterpene indole alkaloid psychollatine and the crude foliar extract of *Psychotria umbellata* Vell. **Toxicology in Vitro 22:** 559-566.

Frohnmeyer, H. & Staiger, D. 2003. Ultraviolet-B radiation-mediated responses in plants. Balancing damage and protection. **Plant Physiology 133:** 1420-1428.

Gao, Q. & Zhang, L. 2008. Ultraviolet-B-induced oxidative stress and antioxidant defense system responses in ascorbate-deficient *vtc1* mutants of *Arabidopsis thaliana.* **Journal of Plant Physiology 165:** 138-148.

Gechev, T. S. & Hille, J. 2005. Hydrogen peroxide as a signal controlling plant cell death. **Journal of Cell Biology 168(1):** 17-20.

Gould, K. S.; McKelvie, J.; Markham, K. R. 2002. Do anthocyanins function as antioxidant in leaves? Imaging of H_2O_2 in red and green leaves after mechanical damage. **Plant, Cell and Environment 25:** 1261-1269.

Gregianini, T. S.; Silveira, V. C.; Porto, D. D.; Kerber, V. A.; Henriques, A. T.; Fett-Neto, A. G. 2003a. The alkaloid brachycerine is induced by ultraviolet radiation and is a singlet oxygen quencher. **Photochemistry and Photobiology 78**(5): 470-474.

Gregianini, T. S. 2003b. Produção do alcalóide bioativo braquicerina em *Psychotria brachyceras* Müller Argoviensis (Rubiaceae). Tese de Doutorado, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS.

Gregianini, T. S.; Porto, D. D.; Nascimento, N. C.; Fett, J. P.; Henriques, A. T.; Fett-Neto, A. G. 2004. Environmental and ontogenetic control of accumulation of brachycerine, a bioactive indole alkaloid from *Psychotria brachyceras.* **Journal of Chemical Ecology 30(10):** 2023-2036.

Gurib-Fakim, A. 2005. Medicinal plants: traditions of yesterday and drugs of tomorrow. **Molecular Aspects of Medicine 27(1):** 1-93.

Harborne, J. 1993. Introduction to Ecological Biochemistry. 4ª ed., Academic Press, Londres.

Harborne, J. B. & Williams, C. A. 2000. Advances in flavonoid research since 1992. **Phytochemistry 55:** 481-504.

Hartmann, T. 2007. From waste products to ecochemicals: fifty years research of plant secondary metabolism. **Phytochemistry 68:** 2831-2846.

Harvey, A. 2000. Strategies for discovering drugs from previously unexplored natural products. **Drug Discovery Ltd. 5(7):** 294-300.

He, Y. & Häder, D. 2002. UV-B-induced formation of reactive oxygen species and oxidative damage of the cyanobacterium *Anabaena* sp.: protective effects of ascorbic acid and *N*-acetyl-L-cysteine. **Journal of Photochemistry and Photobiology B: Biology 66:** 115-124.

Henriques, A. T.; Lopes, S. O.; Paranhos, J. T.; Gregianini, T. S.; Poser, G. L. V.; Fett-Neto, A. G.; Schripsema, J. 2004. N,β-D-Glucopyranosil vincosamide, a light regulated indole alkaloid from the shoots of *Psychotria leiocarpa.* **Phytochemistry 65:** 449-454.

Hideg, É.; Barta, C.; Kálai, T.; Vass, I.; Hideg, K.; Asada, K. 2002. Detection of singlet oxygen and superoxide with fluorescent sensors in leaves under stress by photoinhibition or UV radiation. **Plant & Cell Physiology 43(10):** 1154-1164.

Hollósy, F. 2002. Effects of ultraviolet radiation on plant cells. **Micron 33:** 179-197.

Huey, R. B.; Carlson, M.; Crozier, L.; Frazier, M.; Hamilton, H.; Harley, H.; Hoang, A.; Kingsolver, J. G. 2002. Plants *versus* animals: do they deal with stress in different ways? **Integrative and Comparative Biology 42:** 415-423.

Jansen, M. A. K.; Gaba, V.; Greenberg, B. M. 1998. Higher plants and UVB radiation: balancing damage, repair and acclimation. **Trends in Plant Science 3(4):** 131-135.

Jansen, M. A. K.; Hectors, K.; O'Brien, N. M.; Guisez, Y.; Potters, G. 2008. Plant stress and human health: Do human consumers benefit from UV-B acclimated crops? **Plant Science 175:** 449-458.

Julkunen-Tiito, R.; Häggman, H.; Aphalo, P. J.; Lavola, A.; Tegelberg, R.; Veteli, T. 2005. Growth and defense in deciduous trees and shrubs under UV-B. **Environmental Pollution 137:** 404-414.

Kamiya, K.; Fujita, Y.; Saiki, Y.; Hanani, E.; Mansur, U.; Satake, T. 2002. Studies on the constituents of indonesian *Borreria latifolia*. **Heterocycles 56:** 537-543.

Karban, R. & Myers, J. 1989. Induced plant responses to herbivory. **Annual Review in Ecology and Systematics 20:** 331-348.

Kerber, V. A.; Gregianini, T. S.; Paranhos, J.; Schwambach, J.; Farias, F.; Fett, J. P.; Fett-Neto, A. G.; Zuanazzi, J. A. S.; Quirion, J.; Elisabetsky, E.; Henriques, A. T. 2001. Brachycerine, a novel monoterpene indole alkaloid from *Psychotria brachyceras.* **Journal of Natural Products 64(5):** 677-679.

Kerber, V. A.; Passos, C. S.; Verli, H.; Fett-Neto, A. G.; Quirion, J. P.; Henriques, A. T. 2008. Psychollatine, a glucosidic monoterpene indole alkaloid from *Psychotria umbellata*. **Journal of Natural Products 71:** 697-700.

Khan, M. B. & Harborne, J. B. 1991. A comparison of the effect of mechanical and insect damage on alkaloid levels in *Atropa acuminata.* **Biochemical Systematics and Ecology 19(7):** 529-534.

Kytridis, V. & Manetas, Y. 2006. Mesophyll versus epidermal anthocyanins as potential *in vivo* antioxidants: evidence linking the putative antioxidant role to the proximity of oxy-radical source. **Journal of Experimental Botany 57(10):** 2203-2210.

Knust, L. & Samuels, A. L. 2003. Biosynthesis and secretion of plant cuticular wax. **Progress in Lipid Research 42:** 51-80.

Koo, B. & Wright, B. D. 1999. The role of biodiversity products as incentives for conserving biological diversity: some instructive examples. **The Science of the Total Environment 240:** 21-30.

Lameira, J.; Alves, C. N.; Moliner, V.; Silla, E. 2006. A density functional study of flavonoid compounds with anti-HIV activity. **European Journal of Medical Chemistry 41(5):** 616-623.

Landry, L. G.; Chapple, C. C. S.; Last, R. G. 1995. Arabidopsis mutants lacking phenolic sunscreens exhibit enhanced ultraviolet-B injury and oxidative damage. **Plant Physiology 109:** 1159-1166.

Leal, M. B. & Elisabetsky E. 1996. Absence of alkaloids in *Psychotria carthagenensis* Jacq. (Rubiaceae). **Journal of Ethnopharmacology 54:** 37-40.

Li, J.; Ou-Lee, T.; Raba, R.; Amundson, R. G.; Last, R. G. 1993. *Arabidopsis* flavonoid mutants are hypersensitive to UV-B radiation. **Plant Cell 5:** 171-179.

Limberger, R. P.; Aleixo, A. M.; Fett-Neto, A. G.; Henriques, A. T. 2007. Bioconversion of (+)- and (-)-alpha pinene to (+)- and (-)-verbenone by plant cell cultures of *Psychotria brachyceras* and *Rauvolfia sellowii*. **Eletronic Journal of Biotechnology 10(4):** 500-507.

Magnani, L.; Gaydou, E. M.; Hubaud, J. C. 2000. Spectrophotometric measurement of antioxidant properties of flavones and flavonols against superoxide anion. **Analytica Chimica Acta 411:** 209-216.

Maimone, T. J. & Baran, P. S. 2007. Modern synthethic efforts toward biologically active terpenes. **Nature Chemical Biology 3(7):** 396-407.

Malanga, G.; Kozak, R. G.; Puntarulo, S. 1999. N-Acetylcysteine-dependent protection against UV-B damage in two photosynthetic organisms. **Plant Science 141:** 129-137.

Manetas, Y. 2006. Why some leaves are anthocyanic and why most anthocyanic leaves are red? **Flora 201:** 163-177.

Mayser, P.; Schäfer, U.; Krämer, H.; Irlinger, B.; Steglich, W. 2002. Pityriacitrin – an ultraviolet-absorbing indole alkaloid from the yeast *Malassezia furfur.* **Archives of Dermatological Research 294:** 131-134.

Memelink, J.; Verpoorte, R.; Kijne, J. W. 2001. ORCAnization of jasmonate-responsive gene expression in alkaloid metabolism. **Trends in Plant Science 6(5):** 212-219.

Middleton, E. M. & Teramura, A. H. 1993. The role of flavonol glycosides and carotenoids in protecting soybean from ultraviolet-B damage. **Plant Physiology 103:** 741-752.

Murata, J.; Roepke, J.; Gordon, H.; De Luca, V. 2008. The leaf epidermome of *Catharanthus roseus* reveals its biochemical specialization. **Plant Cell 20:** 524-542.

Nascimento, N. C., Fragoso, V., Moura, D. J., Silva, A. C. R., Fett-Neto, A. G., Saffi, J. 2007. Antioxidant and antimutagenic effects of the crude foliar extract and the alkaloid Brachycerine of *Psychotria brachyceras.* **Environmental and Molecular Mutagenesis 48(9):** 728-734.

Nepokroeff, M.; Bremer, B.; Systma, K. J. 1999. Reorganization of the genus *Psychotria* and tribe Psychotrieae (Rubiaceae) inferred from ITS and *rbcL* sequence data. **Systematic Botany 24(1):** 5-27.

Orozco-cardenas, M. & Ryan, C. 1999. Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. **Proceedings of the National Academy of Sciences of the USA 96:** 6553-6557.

Pasquali, G.; Porto, D. D.; Fett-neto, A. G. 2006. Metabolic engineering of cell cultures versus whole plant complexity in production of bioactive monoterpene indole alkaloids: recent progress related to old dilemma. **Journal of Bioscience and Bioengineering 101(4):** 287-296.

Pilon, J. J.; Lambers, H; Baas, W.; Tosserams. M.; Rozema, J.; Atkin, O. K. 1999. Leaf waxes of slow-growing alpine and fast-growing lowland *Poa* species: inherent differences and responses to UV-B radiation. **Phytochemistry 50:** 571-580.

Rates, S.M.K. 2001. Plants as source of drugs. **Toxicon 39:** 603-613.

Rice-Evans, C. A.; Miller, N. J.; Paganga, G. 1997. Antioxidant properties of phenolic compounds. Trends in Plant Science 2: 152-159.

Sagi, M.; Davydov, O.; Orazova, S.; Yesbergenova, Z.; Ophir, R.; Stratmann, J. W.; Fluhr, R. 2004. Plant respiratory burst oxidase homologs impinge on wound responsiveness and development in *Lycopersicon esculentum.* **Plant Cell 16:** 616-628.

Santos, L. V.; Fett-Neto, A. G.; Kerber, V. A.; Elisabetsky, E.; Quirion, J. C.; Henriques, A. T. 2001. Indole monoterpene alkaloids from leaves of *Psychotria suterella* Müll. Arg. (Rubiaceae). **Biochemical Systematics and Ecology 29:** 1185-1187.

Schweitzer, C. & Schmidt, R. 2003. Physical mechanisms of generation and deactivation of singlet oxygen. **Chemical Reviews 103:** 1685-1757.

Siciliano, T., De Leo, M., Bader, A., De Tommasi, N., Vrieling, K., Braça, A., Morelli, I. 2005. Pyrrolizidine alkaloids from *Anchusta strigosa* and their antifeedant activity. **Phytochemistry 66:** 1593-1600.

Simões-Pires, C. A.; Farias, F. M.; Marston, A.; Queiroz, E. F.; Chaves, C. G.; Henriques, A. T.; Hostettman, K. 2006. Indole monoterpenes with antichemotactic activity from *Psychotria myriantha*: chemotaxonomic significance. **Natural Product Communications 1(12):** 1101-1106.

Smith, J. L.; Burrit, D. J.; Bannister, P. 2000. Shoot dry weight, chlorophyll and UV-B-absorbing compounds as indicators of a plant's sensitivity to UV-B radiation. **Annals of Botany 86:** 1057-1063.

Šnyrychová, I.; Kós, P. B.; Hideg, É. 2007. Hydroxyl radicals are not the protagonists of UV-B induced damage in isolated thylakoid membranes. **Functional Plant Biology 34:** 1112-1121.

Šprtová, M.; Marek, M. V.; Nedbal, L; Prášil, O.; Kalina, J. 1999. Seasonal changes of photosynthetic assimilation of Norway spruce under the impact of UV-B radiation. **Plant Science 142:** 37-45.

Stowe, K. A.; Marquis, R. J.; Hochwender, C. G.; Simms, E. L. 2000. The evolutionary ecology of tolerance to consumer damage. **Annual Review in Ecology and Systematics 31:** 565-95.

Strauss, S. Y. & Agrawal, A. A. 1999. The ecology and evolution of plant tolerance to herbivory. **Tree 14(5):** 179-185.

Taiz, L. & Zeiger, E. 1999. Fisiologia Vegetal. Artmed, Porto Alegre, 3ª ed.

Thordal-Christensen, H.; Zhang, Z.; Wei, Y.; Collinge, D. B. 1997. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during barley-powdery mildew interaction. **Plant Journal 11(6):** 1187-1194.

Tomizawa, M. & Casida, J. E. 2003. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. **Annual Review in Entomology 48:** 339-364.

Turlings, T. C. J.; Loughrin, J. H.; McCall, P. J.; Röse, U. S. R.; Lewis, W. J.; Tumlinson, J. H. 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. **Proceedings of the National Academy of Sciences of USA 92:** 4169-4174.

Van Breusegem, F.; Vranová, E.; Dat, J. F.; Inzé, D. 2001. The role of active oxygen species in plant signal transduction. **Plant Science 161:** 405-414.

Van Rensen, J. J. S.; Vredenberg, W. J.; Rodrigues, G. C. 2007. Time sequence of the damage to the acceptor and donor sides of photosystem II by UV-B radiation as evaluated by chlorophyll *a* fluorescence. **Photosynthesis Research 94:** 291-297.

Van Schie, C. C. N.; Haring, M. A.; Schuurink, R. C. 2006. Regulation of terpenoid and benzenoid production in flowers. **Current Opinion in Plant Biology 9:** 203-208.

Velozo, J. A.; Alvarez, R. I.; Wächter, G. A.; Timmerman, B. N.; Corcuera, L. J. 1999. Increase in gramine content in barley infested with the aphid *Schizaphis graminum* R. **Phytochemistry 52(6):** 1059-1061.

Verotta, L.; Pilati, T.; Tatò, M.; Elisabetsky, E.; Amador, T. A.; Nunes, D. S. 1998. Pyrrolidinoindoline alkaloids from *Psychotria colorata*. **Journal of Natural Products 61:** 392-396.

Verotta, L.; Peterlongo, F.; Elisabetsky, E.; Amador, T. A.; Nunes, D. S. 1999. High-performance liquid chromatography–diode array detection–mass spectrometry analyses of the alkaloid extracts of Amazon *Psychotria* species. **Journal of Chromatography A 841:** 165-176.

Vilariño, M. P.; Mareggiani, G.; Grass, M. Y.; Leicach, S. R.; Ravetta, D. A. 2005. Post-damage alkaloid concentration in sweet and bitter lupin varieties and its effect in subsequent herbivory. **Journal of Applied Entomology 129:** 233-279.

Vilariño, M. P. & Ravetta, D. A. 2008. Tolerance to herbivory in lupin genotypes with different alkaloid concentration: interspecific differences between *Lupinus albus* L. and *L. angustifolius* L. **Environmental and Experimental Botany 63:** 130-136.

Wasternack, C. 2007. Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. **Annals of Botany 100:** 681-697.

WHO, World Health Organization, http://www.who.int/medicines/publications/08_ENGLISH_indexFINAL_EML15.pdf

Wilson, R. M. & Danishefsky, S. J. 2006. Small molecule natural products in the discovery of therapeutic agents: the synthesis connection. **Journal of Organic Chemistry 71:** 8329-8351.

Yanqun, Z.; Yuan, L.; Haiyan, C.; Jianjun, C. 2003. Intraspecific differences in physiological response of 20 soybean cultivars to enhanced UV-B radiation under field conditions. **Environmental and Experimental Botany 50:** 87-97.

Yuan, L.; Yanqun, Z.; Jianjun, C.; Haiyan, C.; Jilong, Y.; Zhide, H. 2000. Intraspecific differences in physiological response of 20 wheat cultivars to enhanced UV-B radiation under field conditions. **Environmental and Experimental Botany 44:** 95-103.

Zavala, J. A.; Patankar, A. G.; Gase, K.; Baldwin, I. T. 2004. Constitutive and inducible trypsin protease inhibitor production incurs large fitness costs in *Nicotiana attenuata.* **Proceedings of the National Academy of Sciences of the USA 101(6):** 1607-1612.

ANEXO I: Metodologia para obtenção de braquicerina autêntica

Para obtenção de braquicerina autêntica foi seguido o protocolo usado por Kerber (1999), com algumas modificações. O material vegetal, folhas totalmente expandidas, foi coletado no Morro Santana (Campus do Vale – UFRGS) e seco à sombra por no mínimo uma semana. O extrato etanólico bruto foi obtido por maceração com etanol. A aproximadamente 400g de folhas moídas em liquidificador, foram adicionados 3 litros de etanol comercial. A suspensão foi macerada (mantida à temperatura ambiente no escuro) por uma semana com agitação ocasional. A suspensão foi filtrada à vácuo usando papel filtro qualitativo e o material vegetal foi reextraído em etanol novo da mesma maneira por mais duas semanas. Os extratos etanólicos foram reunidos e concentrados em evaporador rotatório a vácuo sob temperatura não superior a 40°C até apresentarem consistência xaroposa e não ter sido detectado odor alcoólico. O extrato foi dissolvido em HCl 0,1 M e lavado com CH_2Cl_2. Posteriormente, a fração aquosa foi alcalinizada com NH_4OH 25% até pH=10 e os alcalóides foram extraídos com CH_2Cl_2 em soxhlet modificado para este fim até reação de Dragendorff negativa.

O extrato alcaloídico bruto obtido foi submetido à cromatografia em coluna flash usando sílica como fase estacionária (Sílica gel 60, tamanho de grão 0,063 a 0,2 mm, 70 – 230 mesh, Macherey-Nagel, Düren, Alemanha). Foi utilizada a proporção amostra:sílica de 1:100. O eluente inicial consistiu em $CHCl_3$:MeOH (95:5) com adição gradativa MeOH, como eluente (90:10, 85:15, 80:20, 75:25, 70:30 e 50:50, no volume de 500 ml cada). As frações resultantes foram analisadas por cromatografia em camada delgada em placa de sílica usando $CHCl_3$:MeOH (8:2) como fase móvel. As frações que apresentaram maior pureza foram analisadas por CLAE com arranjo de fotodiodo (Shimadzu, Kyoto, Japão). As frações 70:30 e 50:50 ($CHCl_3$:MeOH) forneceram braquicerina autêntica.

ANEXO II: Curriculum vitae

1. DADOS PESSOAIS

Nome: Diogo Denardi Porto
Nome em publicações: Porto, D. D.
Filiação: Vilma Olga Denardi Porto e Pedro Osório Ferreira Porto
Nascimento: 04/12/1980 em Porto Alegre – RS – Brasil
Identidade: 1071220774 SSP-RS
endereço eletrônico: diogodporto@yahoo.com.br, diogodporto@hotmail.com

Endereço Profissional: Laboratório de Fisiologia Vegetal
Departamento de Botânica, IB
Universidade Federal do Rio grande do Sul
Av. Bento Gonçalves, 9500
Prédio 43423, sala 205
Porto Alegre, RS, CEP 91501-970
(55) 51 3308 7637
http://www.cbiot.ufrgs.br/fisiovegetal

2. FORMAÇÃO ACADÊMICA/TITULAÇÃO

2.1 Graduação:

1999-2003: Graduação em Bacharelado em Ciências Biológicas, Ênfase em Biologia Celular e Molecular

3. ESTÁGIOS

3.1 03/2000 – 03/2004: Bolsista de Iniciação Científica do CNPq
Laboratório de Fisiologia Vegetal
Departamento de Botânica, IB

Universidade Federal do Rio grande do Sul, Porto Alegre, RS, Brasil
Orientador: Prof. Dr. Arthur Germano Fett-Neto

3.2 2003: Estágio Supervisionado Curricular
Laboratório Central – Fundação Estadual de Produção e Pesquisa em Saúde
Av. Ipiranga, 5400, Porto Alegre, Rio Grande do Sul, Brasil
Supervisora: Dra. Tatiana Schaffer Gregianini

4. BOLSAS

4.1 Modalidade: Bolsista de Iniciação Científica CNPq
Área do conhecimento: Botânica
Instituição: Universidade Federal do Rio Grande do Sul
Início: 08/2000
Término: 03/2004

4.2 Modalidade: Bolsista de Mestrado CNPq
Área do conhecimento: Biotecnologia
Instituição: Universidade Federal do Rio Grande do Sul
Início: 04/2004
Término: 03/2005

4.3 Modalidade: Bolsista de Doutorado CAPES
Área do conhecimento: Biotecnologia
Instituição: Universidade Federal do Rio Grande do Sul
Início: 04/2005
Término: 03/2009

5. MONOGRAFIA DE CONCLUSÃO DE CURSO

Título: Influência da Radiação Ultravioleta e do Dano Mecânico na Concentração do Alcalóide braquicerina em Folhas de *Psychotria brachyceras* Mull. Arg.

Orientador: Prof. Dr. Arthur Germano Fett-Neto
Laboratório de Fisiologia Vegetal, Departamento de Botânica e Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brasil.

6. ARTIGOS CIENTÍFICOS PUBLICADOS

6.1 Gregianini, T. S.; Silveira, V. C.; **Porto, D. D**.; Kerber, V. A.; Henriques, A. T.; Fett-Neto, A. G. 2003a. The alkaloid brachycerine is induced by ultraviolet radiation and is a singlet oxygen quencher. **Photochemistry and Photobiology 78(5):** 470-474.

6.2 Gregianini, T. S.; **Porto, D. D.**; Nascimento, N. C.; Fett, J. P.; Henriques, A. T.; Fett-Neto, A. G. 2004. Environmental and ontogenetic control of accumulation of brachycerine, a bioactive indole alkaloid from *Psychotria brachyceras.* **Journal of Chemical Ecology 30(10):** 2023-2036.

6.3 Pasquali, G.; **Porto, D. D**.; Fett-Neto, A. G. 2006. Metabolic engineering of cell cultures versus whole plant complexity in production of bioactive monoterpene indole alkaloids: recent progress related to old dilemma. **Journal of Bioscience and Bioengineering 101(4):** 287-296.

7. TRABALHOS COMPLETOS PUBLICADOS EM ANAIS DE EVENTOS

7.1 PORTO, D. D. . Induction of alkaloid biosynthesis in cuttings of Psychotria brachyceras (Rubiaceae) by UV radiation. In: XI Reunion Latinoamericana de Fisiología Vegetal, 2002, Punta del Este. Actas, 2002. p. 201-202.

7.2 PARANHOS, J. T. ; GREGIANINI, T. S. ; SCHWAMBACH, J. ; **PORTO, D. D.** ; FRAGOSO, V. ; CAMARGO, F. ; FETT, J. P. ; FETTNETO, A. G. ; ZUANAZZI, J. A. ; HENRIQUES, A. T. . Rizogênese adventícia em espécies de Psychotria (Rubiaceae) do sul do Brasil produtoras de alcalóides bioativos. In: VIII Congresso Brasileiro de Fisiologia Vegetal, 2001, Ilhéus. Anais., 2001. p. 76.

8. RESUMOS PUBLICADOS EM ANAIS DE EVENTOS

8.1 PORTO, D. D. ; SANTOS, D. A. ; NERY, L. ; PEDROLLO, C. ; FETTNETO, A. G. UV-B Radiation Responses in Two Psychotria Species. In: XI Congresso Brasileiro de Fisiologia Vegetal, 2007, Gramado. Livro de Resumos.

8.2 PORTO, D. D. ; SANTOS, D. A. ; FETTNETO, A. G. . Respostas de Psychotria brachyceras Müll. Arg. (Rubiaceae) à Radiação Ultravioleta: Acúmulo de Braquicerina e Flavonóides.. In: 57º Congresso Nacional de Botânica, 2006, Gramado. CD Resumos do Congresso.

8.3 PORTO, D. D. ; SANTOS, D. A. ; HENRIQUES, A. T. ; FETTNETO, A. G. . brachycerine production in *Psychotria brachyceras*: relation with fruit setting and phenolics accumulation upon wounding. In: X Congresso Brasileiro de Fisiologia Vegetal, 2005, Recife. Livro de resumos.

8.4 PORTO, D. D. ; SANTOS, D. A. ; HENRIQUES, A. T. ; FETTNETO, A. G. . Brachycerine production in Psychotria brachyceras: Relation with fruit setting and phenolics accumulation upon wounding. In: VII Reunião Anual do Programa de Pós-graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS, 2005, Porto Alegre. Livro de Resumos, p. 76.

8.5 PORTO, D. D. ; GREGIANINI, T. S. ; NASCIMENTO, N. C. ; FETTNETO, A. G. . A Biossíntese de Braquicerina, um Alcalóide Monoterpeno Indólico Bioativo de Psychotria brachyceras, é Regulada por Jasmonato. In: 55 Congresso Nacional de Botânica, 2004, Viçosa. Livro de Resumos.

8.6 GREGIANINI, T. S. ; **PORTO, D. D.** ; HENRIQUES, A. T. ; FETTNETO, A. G. . Organ-Specific and developmental control of accumulation of brachycerine, a terpenoid indole alkaloid of Psychotria brachyceras. In: IX Congresso Brasileiro de Fisiologia Vegetal, 2003, Atibaia. Caderno de Resumos, v. 15. p. 22-22.

8.7 GREGIANINI, T. S. ; **PORTO, D. D.** ; NASCIMENTO, N. C. ; HENRIQUES, A. T. ; FETTNETO, A. G. . Influence of stress factors on the accumulation of brachycerine, a bioactive indole alkaloid from Psychotria brachyceras. In: IX Congresso Brasileiro de Fisiologia Vegetal, 2003, Atibaia. Livro de Resumos, v. 15. p. 30-30.

8.8 GREGIANINI, T. S. ; PARANHOS, J. T. ; **PORTO, D. D**. ; FRAGOSO, V. ; FETT, J. P. ; FETTNETO, A. G. . Efeito da herbivoria simulada em folhas de Psychotria brachyceras e P. umbellata na produção de alcalóides bioativos. In: Congresso Nacional de Botânica, 2002, Fortaleza. Anais.

8.9 GREGIANINI, T. S. ; PARANHOS, J. T. ; **PORTO, D. D.** ; FRAGOSO, V. ; FETT, J. P. ; FETTNETO, A. G. . Efeito da herbivoria simulada em folhas de Psychotria brachyceras e P. umbellata na produção de alcalóides bioativos. In: Congresso Nacional de Botânica, 2002, Fortaleza. Anais.

8.10 GREGIANINI, T. S. ; PARANHOS, J. T. ; SCHWAMBACH, J. ; **PORTO, D. D.** ; FRAGOSO, V. ; CAMARGO, F. ; FETT, J. P. ; FETTNETO, A. G. ; ZUANAZZI, J. A. ; HENRIQUES, A. T. . Rizogênese adventícia em espécies de Psychotria

produtoras de alcalóides bioativos. In: III Reunião Anual do Programa de Pós-graduação em biologia celular e molecular, 2001, Porto Alegre. Livro de Resumos. Editora da Universidade, p. 34.

8.11 GREGIANINI, T. S. ; **PORTO, D. D**. ; FETTNETO, A. G. ; FETT, J. P. . Influência da Nutrição mineral e do dano mecânico no acúmulo de alcalóides bioativos em folhas de Psychotria brachyceras. In: III Reunião Anual do Programa de Pós-graduação em biologia celular e molecular, 2001, Porto Alegre. Livro de Resumos. Editora da Universidade, p. 35.

8.12 PORTO, D. D. ; GREGIANINI, T. S. ; FETT, J. P. ; FETTNETO, A. G. . Regulação da biossíntese do alcalóide braquicerina em plantas de Psychotria brachyceras. In: XIII Salão de Iniciação Científica - UFRGS, 2001, Porto Alegre. Programa e Resumos. Porto Alegre : Editora da Universidade.

8.13 GREGIANINI, T. S. ; PARANHOS, J. T. ; SCHWAMBACH, J. ; **PORTO, D. D.** ; FRAGOSO, V. ; CAMARGO, F. ; FETT, J. P. ; FETTNETO, A. G. ; ZUANAZZI, J. A. ; HENRIQUES, A. T. . Rizogênese adventícia em espécies de Psychotria produtoras de alcalóides bioativos. In: III Reunião Anual do Programa de Pós-graduação em biologia celular e molecular, 2001, Porto Alegre. Livro de Resumos. Editora da Universidade, p. 34.

8.14 GREGIANINI, T. S. ; **PORTO, D. D.** ; FETTNETO, A. G. ; FETT, J. P. . Influência da Nutrição mineral e do dano mecânico no acúmulo de alcalóides bioativos em folhas de Psychotria brachyceras. In: III Reunião Anual do Programa de Pós-graduação em biologia celular e molecular, 2001, Porto Alegre. Livro de Resumos. Porto Alegre : Editora da Universidade p. 35.

8.15 PORTO, D. D. ; GREGIANINI, T. S. ; FETT, J. P. ; FETTNETO, A. G. . Regulação da biossíntese do alcalóide braquicerina em plantas de Psychotria

brachyceras. In: XIII Salão de Iniciação Científica - UFRGS, 2001, Porto Alegre. Programa e Resumos. Editora da Universidade.

8.16 MANDAJI, M. ; OLIVEIRA, A. P. ; **PORTO, D. D**. ; FETTNETO, A. G. ; FETT, J. P. . Mecanismos de resistência ao excesso de ferro em arroz: estudos preliminares. In: II Reunião Anual do Programa de Pós-graduação em biologia celular e molecular, 2000, Porto Alegre. Livro de Resumos. Editora da Universidade, p. 34.

8.17 OLIVEIRA, A. P. ; **PORTO, D. D**. ; MANDAJI, M. ; FETTNETO, A. G. ; FETT, J. P. . Exclusão como mecanismo de resistência ao excesso de ferro em arroz. In: XII Salão de Iniciação Científica, 2000, Porto Alegre. Anais. Editora da Universidade.

8.18 FETTNETO, A. G. ; **PORTO, D. D**. ; GREGIANINI, T. S. ; GOULART, L. W. ; HENRIQUES, A. T. . Cultura in vitro de Psychotria spp. produtoras de alcalóides bioativos. In: Congresso Nacional de Botânica, 2000, Brasília, DF. Programa e Resumos, p. 51.

8.19 GREGIANINI, T. S. ; **PORTO, D. D.** ; FARIAS, F. ; ZUANAZZI, J. A. ; FETTNETO, A. G. ; HENRIQUES, A. T. . Regulação do acúmulo de N,B-D-Glicopiranosilvincosamida, alcalóide monoterpeno indólico de Psychotria leiocarpa (Rubiaceae).. In: 46º Congresso Nacional de Genética, 2000, Águas de Lindóia, SP. Genetics and Molecular Biology, v. 23. p. 468.

8.20 MANDAJI, M. ; OLIVEIRA, A. P. ; **PORTO, D. D.** ; FETTNETO, A. G. ; FETT, J. P. . Mecanismos de resistência ao excesso de ferro em arroz: estudos preliminares. In: II Reunião Anual do Programa de Pós-graduação em biologia celular e molecular, 2000, Porto Alegre. Livro de Resumos. Editora da Universidade, p. 34.

8.21 OLIVEIRA, A. P. ; **PORTO, D. D.** ; MANDAJI, M. ; FETTNETO, A. G. ; FETT, J. P. . Exclusão como mecanismo de resistência ao excesso de ferro em arroz. In: XII Salão de Iniciação Científica, 2000, Porto Alegre. Anais. Editora da Universidade.

8.22 FETTNETO, A. G. ; **PORTO, D. D.** ; GREGIANINI, T. S. ; GOULART, L. W. ; HENRIQUES, A. T. . Cultura in vitro de Psychotria spp. produtoras de alcalóides bioativos. In: Congresso Nacional de Botânica, 2000, Brasília, DF. Programa e Resumos, p. 51.

8.23 GREGIANINI, T. S. ; **PORTO, D. D**. ; FARIAS, F. ; ZUANAZZI, J. A. ; FETTNETO, A. G. ; HENRIQUES, A. T. . Regulação do acúmulo de N,B-D-Glicopiranosilvincosamida, alcalóide monoterpeno indólico de Psychotria leiocarpa (Rubiaceae).. In: 46º Congresso Nacional de Genética, 2000, Águas de Lindóia, SP. Genetics and Molecular Biology, v. 23. p. 468.

ANEXO III: Artigo publicado durante o doutoramento

Metabolic engineering of cell cultures versus whole plant complexity in production of bioactive monoterpene indole alkaloids: recent progress related to old dilemma.

JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 101, No. 4, 287–296. 2006
DOI: 10.1263/jbb.101.287

REVIEW

Metabolic Engineering of Cell Cultures versus Whole Plant Complexity in Production of Bioactive Monoterpene Indole Alkaloids: Recent Progress Related to Old Dilemma

Giancarlo Pasquali,[1,2] Diogo Denardi Porto,[1,3] and Arthur Germano Fett-Neto[1,3]*

Programa de Pós-graduação em Biologia Celular e Molecular, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9500, Pr. 43.431, P.O. Box 15.005, CEP 91.501-970, Porto Alegre, RS, Brazil,[1] Departamento de Biologia Molecular e Biotecnologia, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil,[2] and Departamento de Botânica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil[3]

Received 6 October 2005/Accepted 12 November 2005

Monoterpene indole alkaloids (MIAs) are a large class of plant alkaloids with significant pharmacological interest. The sustained production of MIAs at high yields is an important goal in biotechnology. Intensive effort has been expended toward the isolation, cloning, characterization and transgenic modulation of genes involved in MIA biosynthesis and in the control of the expression of these biosynthesis-related genes. At the same time, considerable progress has been made in the detailed description of the subcellular-, cellular-, tissue- and organ-specific expressions of portions of the biosynthetic pathways leading to the production of MIAs, revealing a complex picture of the transport of biosynthetic intermediates among membrane compartments, cells and tissues. The identification of the particular environmental and ontogenetic requirements for maximum alkaloid yield in MIA-producing plants has been useful in improving the supply of bioactive molecules. The search for new bioactive MIAs, particularly in tropical and subtropical regions, is continuously increasing the arsenal for therapeutic, industrially and agriculturally useful molecules. In this review we focus on recent progress in the production of MIAs in transgenic cell cultures and organs (with emphasis on *Catharanthus roseus* and *Rauvolfia serpentina* alkaloids), advances in the understanding of *in planta* spatial-temporal expression of MIA metabolic pathways, and on the identification of factors capable of modulating bioactive alkaloid accumulation in nontransgenic differentiated cultures and plants (with emphasis on new MIAs from *Psychotria* species). The combined use of metabolic engineering and physiological modulation in transgenic and wild-type plants, although not fully exploited to date, is likely to provide the sustainable and rational supply of bioactive MIAs needed for human well being.

[**Key words:** secondary metabolism, monoterpene indole alkaloids, metabolic engineering, *Catharanthus*, *Rauvolfia*, *Psychotria*]

Since the early 1980s, *Catharanthus roseus* (L.) G. Don and *Rauvolfia serpentina* (L.) Benth. ex Kurz became plant model systems for the investigation of monoterpene indole alkaloid (MIA) production by *in vitro* culture techniques, particularly cell suspension cultures. However, land-cultivated Madagascar periwinkle or vinca (*C. roseus*) plants are still the only viable commercial source of the anticancer drugs vinblastine and vincristine, as well as serpentine and ajmalicine, the active compounds of available medicines for treating hypertension, arrhythmia and different vascular diseases (1). Likewise, *R. serpentina* field-cultivated plants are still the main source of MIAs such as ajmalicine and ajmaline, also used in the therapy of cardiovascular disorders (2). The chemical synthesis of these highly-complex natural products is generally not feasible or commercially not viable, making MIA-producing plants the sole source of the bioactive molecules.

The lack of knowledge on the fully functional pathways required for the production of target molecules is pointed out as the main reason why the attempts to use plant cell cultures as alternative sources of natural products have been problematic. Nevertheless, more than 130 different MIA structures have been characterized from *C. roseus* and *R. serpentina* (1). Considering the precursors isopentenyl diphosphate and chorismate, at least 24 different specific MIA-

* Corresponding author. e-mail: fettneto@cbiot.ufrgs.br
phone: +55-51-3316-7642 fax: +55-51-3316-7309

synthesizing enzymes have been purified and completely or partially characterized from plants (www.genome.ad.jp/kegg/pathway/map/map00901.html, 2005), and at least 10 genes encoding such enzymes have been cloned (3, 4). Additionally, genes encoding putative transcription factors related to MIA biosynthesis have also been cloned (5). Finally, genomic and proteomic approaches are markedly increasing the number of known genes and enzymes related to MIA biosynthesis (6).

On the other hand, there is increasing awareness that alkaloid biosynthetic pathways are far more than a mere assembly of consecutive enzymes and corresponding regulatory transcription factors, but rather a complex system with highly specialized spatial and temporal controls (at the subcellular, tissue, organ and whole plant levels) in intricate association with biotic and abiotic environmental stimuli, coupled with a tight ontogenetically programmed control of development. Therefore, a detailed understanding of the developmental and ecochemical aspects of alkaloid production in differentiated tissues and whole plants offers important resources for the sustained and commercially viable production of bioactive molecules of interest, both in wild-type and transgenic plants (7).

The chemical, biological and pharmacological characterizations of MIAs and other classes of alkaloids are far from being completed. Only a fraction of the known plant species has been chemically investigated, not to mention the many tropical species presently unknown to science. Recently, a combined ethnobotanical and chemotaxonomical approach has unveiled a new crop of bioactive MIAs from *Psychotria* sp. of South America, some of which showing novel biosynthetic possibilities (8, 9).

In this review we focus on recent progress in the production of MIAs in transgenic cell cultures and organs (with emphasis on *C. roseus* and *R. serpentina* alkaloids), advances in the understanding of *in planta* spatial-temporal expression of MIA metabolic pathways, and on the identification of factors capable of modulating bioactive alkaloid accumulation in nontransgenic differentiated cultures and plants (with emphasis on *Psychotria* alkaloids).

STRATEGIES FOR PRODUCTION OF MIAs FOCUSING ON MOLECULAR LEVEL IN MODEL SPECIES *C. ROSEUS* AND *R. SERPENTINA*

Wild-type *Agrobacterium* genetic transformation *In vitro* root cultures established by wild-type *Agrobacterium rhizogenes*-mediated transformation of leaves, stems or calli have been largely studied as sources of secondary metabolites due to their rapid growth rate and the resulting higher accumulation of such compounds in some of the transformed root lines. Many investigations concerning the establishment of hairy root cultures, the modification of medium constituents and the production of root-specific MIAs have been extensively reviewed (10–12). The more recent study of Batra *et al.* (13) shows well how wild-type *A. rhizogenes*-mediated transformation of leaves allows the generation of *C. roseus* hairy root lines that can accumulate larger amounts of ajmalicine than field-grown roots. The enhanced accumulation of ajmaline and ajmalicine in the hairy roots of *Rauvolfia micrantha* Hook f. compared with untransformed root cultures is also a good recent example of such an approach (2). Hairy roots derived from the wild-type strains of *A. rhizogenes* have also been developed in *Ophiorriza pumila* and *Camptotheca acuminata*, two species capable of producing the anticancer modified MIA camptothecin; the yields of the two species were of approximately 1 mg·g^{-1} dry weight (dw), which were both equivalent to or higher than those obtained with *in planta* roots (reviewed in Ref. 14). Both species were able to partly secrete camptothecin into the medium. For *O. pumila* roots, secretion was stimulated by adding a polystyrene resin to the medium to temporarily bind the alkaloid (15).

The variation in the accumulation of MIAs in roots and cells derived from wild-type *Agrobacterium*-transformed tissues is most probably determined by the induction/repression of MIA-synthesizing genes by *cis* regulatory elements in the T-DNA with enhancer/insulator-like effects. T-DNA copy number and the genomic sites of insertion can account for differences observed among transformed lines. Another possibility to account for such differences would be the existence of as yet uncharacterized T-DNA genes encoding elicitor-like peptides that would promote transcription, or gene products that could mimic the inhibitory effect of auxins on MIA-synthesizing genes. Positional effects on the expression of T-DNA genes would also contribute to the high variability among independent transformed lines.

Clearly, it is possible to obtain *C. roseus* or *Rauvolfia* hairy root and cell lines capable of accumulating higher contents of MIAs by wild-type *Agrobacterium*-mediated transformation. However, it still must be shown if these lines can maintain an improved MIA production over time and on an industrial scale (see below).

Genetic modification of MIA-synthesizing and-controlling genes in aseptic cultures Tryptophan decarboxylase (TDC), strictosidine synthase (STR) and geraniol 10-hydroxylase (G10H) are strongly regulated in *C. roseus* plants and cell suspension cultures. Along with strictosidine glucosidase (SGD), they represent the best characterized MIA- synthesizing enzymes and genes (Fig. 1) (reviewed in Refs. 1, 3, 7, 16–18). In *C. roseus*, the direct over-expression of encoding genes has resulted in the improved accumulation of immediate products, however, in long-term cultures, the accumulation of desirable MIAs was rarely observed. The enhanced activity of recombinant anthranilate synthase (α subunit, ASα) in transformed *C. roseus* hairy roots led to a higher accumulation of tryptophan and tryptamine; however, MIAs remained unchanged (19). This recent finding was not surprising because the overexpression of the *tdc* gene in *C. roseus* calli and cell suspensions was previously demonstrated to result in a higher production of tryptamine, but not MIAs (20–22). Similar results were obtained in *Peganum harmala* (23) and *Cinchona officinalis* (24) but not in *C. roseus* hairy root cultures transformed by the *tdc* gene under the regulation of an inducible promoter (25; see below).

The overexpression of the *str* gene did seem however to enhance serpentine, ajmalicine, catharantine and tabersonine biosyntheses in a number of cell lines (21), although a critical instability has also been observed (22). It became clear

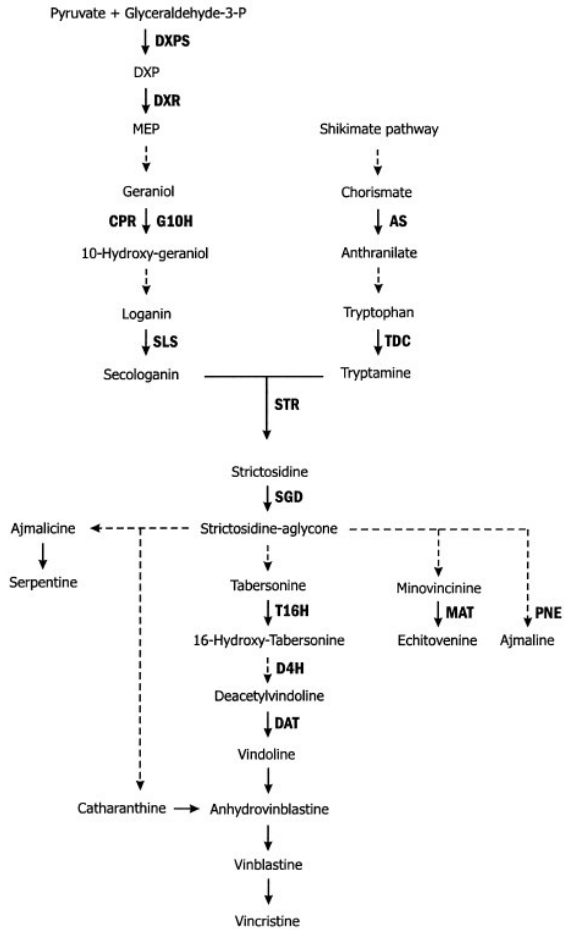


FIG. 1. Metabolic pathways of indole alkaloids (adapted from www.genome.ad.jp/kegg/pathway/map/map00901.html, 2005). The full names of enzymes are presented in the text.

that the presence of the universal precursor strictosidine at higher levels results in a more efficient production of alkaloids. Strictosidine accumulation is dependent on the STR activity and is enhanced by feeding loganin to the transformed cell cultures (21). Recently, *str* gene expression has been shown to be directly correlated to MIA content in plants of different *C. roseus* cultivars and mutants (26). Therefore, and in contrast to the relative independence on TDC activity, MIA accumulation seems to be much more dependent on enzymes of the terpene route.

Whitmer *et al.* (22) showed that the stable *str*- and *tdc*-transformed cell lines of *C. roseus* have decreased recombinant enzyme activities related to MIA biosynthesis and alkaloid accumulation after long-term culture, even under strict selective media. Previously, the same effects were observed with the *str*- and *tdc*-transformed hairy roots of *C. officinalis* (24). Possibly, the cell toxicity of intermediates or MIAs themselves may be the cause of the selection of low- or non-producing lines after long-term culture. Examples of how frequent undesirable effects may occur after transgenic expression of MIA synthesizing genes were presented (27, 28). The authors generated transgenic tobacco plants to examine the effects of localizing TDC to the chloroplast, the site of tryptophan biosynthesis, and to the endoplasmic reticulum, to improve enzyme synthesis. The resulting plants exhibited the highest tryptamine levels reported to date; however, a series of abnormal phenotypes were also observed, particularly necrotic, lesion-mimicking areas in leaves. An interesting strategy to prevent toxicity and improve the yields of MIAs in nontransgenic cell cultures (amenable to application in transformed cell lines) is the protoplast cultures of *C. roseus* immobilized with artificial cell walls made of guluronic acid-rich alginate. When treated with 30 mM calcium chloride, protoplasts remained active for 15 d without cell wall regeneration, releasing alkaloids to the medium and improving the yields of tryptamine, ajmalicine, and catharanthine (29).

As suggested by Verpoorte *et al.* (17), the use of inducible promoters to direct transgene expression may be a feasible approach for improving MIA production in batch cultures. This suggestion has recently been proven correct (25). By employing a glucocorticoid-inducible promoter to drive the expression of the *tdc* gene, an increase of as much as 129% in serpentine specific yields after induction of the *C. roseus* hairy root cultures was obtained. In subsequent experiments, using the same inducible construct of *tdc* in addition to the constitutive expression of one of two forms of ASαβ (wild-type and feedback-intensive anthranilate synthase holoenzyme), significant increases in the yields of ajmalicine, tabersonine and lochnericine were obtained upon induction, depending on the dosage of glucocorticoid and the duration of induction (30).

Besides *asα*, *tdc* and *str*, other available cloned genes encoding MIA-synthesizing enzymes are those for SGD, cytochrome P450-reductase (CPR, acting with geraniol 10-hydroxylase, G10H), G10H, secologanin synthase (SLS), polyneuridine aldehyde esterase (PNE), tabersonine 16-hydroxylase (T16H), desacetoxyvindoline 4-hydroxylase (D4H), deacetylvindoline acetyltransferase (DAT) (4) and minovincinine-19-hydroxy-*o*-acetyltransferase (MAT) (31). However, to the best of our knowledge, no published data are yet available regarding the overexpression of such genes and the resulting effects on MIA biosynthesis in cells, tissues or whole transgenic plants. Among the new candidate genes, the most interesting will be the results of the over-expression of *g10h*, *sls* and other yet uncloned genes of the terpene route, because it has consistently been shown that loganin or secologanin feeding of *C. roseus* cell suspension cultures improves MIA accumulation (reviewed in Refs. 1 and 3). The overexpression of *cpr* will probably affect many other P450-dependent enzymes unrelated to MIA biosynthesis. The genes involved in the biosynthesis of camptothecin have also been cloned from *C. acuminata* or *O. pumila*, including *tdc*, *str* and 10-*hgo* (10-hydroxygeraniol oxidoreductase); however, transgenic modification in root cultures or whole plants has not yet been reported (14). Recently, advances have been made in the modulation of camptothecin yields in the wild-type cell cultures of *C. acuminata* by altering mineral nutrient composition. The increased concentrations of microelements (I, Cu, Co, Mo) resulted in an ap-

proximately 3-fold increase in camptothecin yield, reaching 9.15 mg·l^{-1} (32), whereas a two-stage culture medium supplemented with 70 mM nitrate followed by ammonium/nitrate at a 5:1 ratio (40 mM N) also tripled the yield compared with control cultures (33).

From the studies referred to here and well reviewed by others (34), besides being an excellent approach for better defining the limiting steps in the route leading to the production of MIAs, it is clear that the modulation of single-gene expression is a valid method of improving MIA productivity by *in vitro* cultures, although many limitations still face researchers, such as production instability. Because field-grown plants are still the commercial source of MIAs due to the lower costs of production and particularly because *in vitro* cultures do not yield the high value complex dimeric MIAs, the establishment of efficient protocols for transforming and regenerating whole transgenic plants capable of expressing MIA key transgenes under inducible promoters may prove to be the best strategy for boosting the production of such valuable alkaloids.

The coordinated transcriptional regulation of many MIA-synthesizing genes was observed early in different plant organs and in response to positive signals such as elicitors, UV-light and methyl jasmonate, indicating that a common set of transcription factors would modulate such regulation. The cloning and overexpression of octadecanoid-responsive *Catharanthus* AP2/ERF-domain (ORCA) transcription factors have been shown to regulate the jasmonate-responsive activation of several MIA-synthesizing genes in cell cultures, including *as*α, *tdc*, *cpr*, *str* and *d4h* (5, 35); however, the production of MIAs was not affected unless loganin was fed to the cultures, suggesting that other regulators may act on the control of genes not affected by the ORCA transcription factors. Recently, a new family of zinc finger proteins, named ZCT, has been described in *C. roseus* to have negative effects on *tdc* and *str* genes upon elicitor induction, suggesting that ZCT proteins are repressors in the regulation of elicitor-induced MIA accumulation (36). The demonstration that positive signals such as elicitors and jasmonates trigger the expression of transcriptional activators and the repressors of MIA synthesizing genes clearly indicates how complex MIA regulation is, to a degree that was not expected initially (5, 34). A most interesting experiment concerning the metabolic engineering of MIA-controlling genes would be the overexpression of ORCA genes combined with the repression of the ZCT genes by antisense or RNA interference in *C. roseus* cells.

Finally, the possible impacts of recent advances in genomics and postgenomics over the production of plant secondary metabolites have been recently reviewed (4, 34, 37). Functional genomic approaches such as transcriptomics, proteomics and metabolomics will probably accelerate the identification of new genes involved in secondary metabolite pathways (34). Such techniques when combined are undoubtedly powerful tools for the improvement of secondary product yields by metabolic engineering.

COMPLEXITY OF MIA ACCUMULATION IN WHOLE PLANTS

As can be inferred from the circuitous and transiently effective results of many of the attempts to engineer alkaloid production in cell cultures, the dynamics of assembly of these compounds in plants is rather complex. As pointed out, alkaloid metabolism has been closely associated with specific environmental stimuli as well as with complex ontogenetic control and differentiated organs and tissues. This is one of the reasons that point to genetically modified and/or appropriately treated whole plants as the most promising means of achieving higher-level commercial-scale production of MIAs. Understanding the details of the regulation of alkaloid accumulation is a key aspect for achieving improved yields in plants.

Anatomical and subcellular distribution of MIA biosynthetic pathways The spatial control of alkaloid production in plants is known for various classes of these molecules. In *C. roseus*, the expressions of both mRNA and corresponding proteins involved in major leaf MIA biosynthesis were examined (*tdc*, *str1*, *d4h*, *dat*), indicating that at least two cell types are involved and that there is a need for the translocation of a pathway intermediate (38). *Tdc* and *str1* were localized in the epidermis of stems, leaves and flower buds, and in the protoderm and cortical cells around the apical meristem of root tips. *D4h* and *dat*, involved in the later steps of vindoline biosynthesis, were present in the laticifer and idioblast cells of leaves, stems and flower buds. The same study revealed a basipetal gradient of expression of the four genes (based on *in situ* RNA hybridization experiments) in maturing leaves and in dissected leaves, indicating the transient expression of the vindoline pathway in the early stages of leaf development. The immunocytochemical localization of TDC, D4H and DAT proteins confirmed the differential localization demonstrated by *in situ* hybridization (38). SLS has also been localized in the epidermis of leaves (39). More recently, the coexpression of transcripts from G10H and from three enzymes of the plastidic 2-*C*-methyl-D-erythritol 4-phosphate pathway (MEP pathway) of isopentenyl diphosphate (IPP) biosynthesis has been demonstrated (40). Secologanin has been shown to be produced from IPP synthesized via the MEP pathway in *C. roseus* (41). The transcripts corresponding to G10H, 1-deoxy-D-xylulose 5-phosphate synthase (DXS), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) and 2-*C*-methyl-D-erythritol 2,4 cyclodiphosphate synthase (MECS) were restricted to the internal phloem parenchyma of the young aerial parts of *C. roseus* (40). In this case, a basipetal gradient of expression in young leaves was also observed, decreasing from younger to older parts. These data reinforce the evidence for the need for translocation of intermediates from the internal phloem parenchyma to the epidermis (eventually followed by later transport to idioblasts and laticifers). A few studies have been carried out on the cellular localization of camptothecin and the transcripts involved in its biosynthesis in *C. acuminata* and *O. pumilla*; these data have been recently reviewed in detail (14). It is interesting to observe that in these species, unlike the single copy observed in *C. roseus*, TDC is encoded by a small gene family,

apparently with differential regulation mediated by development and defense against pathogens.

The spatial separation of the specific portions of biosynthetic pathways has also been observed in other classes of alkaloids. In tropane alkaloid biosynthesis, roots are the main site of enzyme activity, with produced alkaloids often being transported to shoots through the xylem. The enzymes of scopolamine biosynthesis are distributed in specific tissues of the primary root in *Hyoscyamus niger*: putrescine-*N*-methyltransferase and hyoscyamine 6β hydroxylase are pericycle-specific, whereas the enzyme acting between these two, tropinone reductase, is localized in the endodermis and outer cortex (42). Regarding benzylisoquinoline alkaloids, distinct cell types are involved in the biosynthesis of related alkaloids. Studies in *Papaver somniferum* showed that, throughout the plant axis, the transcripts of enzymes related to sanguinarine and morphine biosynthesis were localized to companion cells paired to sieve elements in the phloem, whereas the corresponding proteins were restricted to the parietal region of sieve elements adjacent to laticifers, in which the alkaloids accumulate (43). In *Thalictrum flavum*, however, the transcripts of nine consecutive enzymes involved in the assembly of the protoberberine alkaloid (*S*)-canadine had distinct localizations. In roots, gene transcripts for all enzymes were localized in the immature endodermis, pericycle and adjacent cortical cells, whereas in rhizomes transcripts were restricted to the protoderm of leaf primordia (44). The alkaloids resulting from enzyme action accumulated in the mature endodermis of roots upon the onset of secondary growth and throughout the pith and cortex of rhizomes. These results indicate the following: (i) in both *P. somniferum* and *T. flavum*, there is a temporal and spatial separation between alkaloid biosynthesis and accumulation; and (ii) closely related biosynthetic enzymes may be localized in different cell types in *T. flavum* and *P. somniferum*. In addition to new enzyme recruiting, the relocation of established pathways to distinct cell types also appears to be relevant in the evolution of alkaloid metabolism (44).

The spatial control of alkaloid biosynthesis is also observed at the subcellular level. MIAs are a particular alkaloid class in which this feature is evident. The compartmentation of portions of the MIA metabolic pathways of *C. roseus* involves chloroplasts, the cytosol, the endosplasmic reticulum (ER) and vacuoles. The reasons for dividing metabolic pathways to different subcellular compartments include protection against autotoxicity caused by alkaloids and pathway intermediates, better control of metabolic flux (extra regulatory level, reduction of negative feedback) and the optimization of enzyme activity by providing specific requirements in distinct microenvironments. The lack of appropriately differentiated subcellular compartments (*e.g.*, well-developed vacuoles and plastids) in fast-growing cell cultures is likely another reason for the relatively poor biosynthetic performance in these systems. Another aspect that probably contributes to poor MIA accumulation by *in vitro* cultures is the repression of both chloroplast development and the expression of genes involved in photoautotrophic metabolism by the exogenously supplied medium carbohydrates, including those participating in the assembly of the terpenoid moiety of alkaloids (45).

The enzymes of vindoline biosynthesis in *C. roseus* provide a clear example of the rich subcellular compartmentation of MIA biosynthesis (7). The production of tryptamine from tryptophan by TDC occurs in the cytosol; the enzymes of the MEP pathway involved in IPP biosynthesis are located in the chloroplast; G10H involved in one of the steps of secologanin production is localized in provacuolar membranes; STR is inside the vacuole; and SGD is associated with the ER. However, the separation between the cytosolic mevalonate pathway and the plastid MEP pathway is not absolute. Quantitative NMR spectroscopy studies with radiolabeled precursors showed that a simple two-compartment model cannot be used to explain the cross talk between the mevalonate and the MEP pathways of IPP biosynthesis in *C. roseus* cell cultures (46). Studies of the relative contribution of the mevalonate and MEP pathways for supplying IPP units involved in the biosynthesis of MIAs are currently not available for other systems. Subsequent enzymes in the pathway also show compartmentation. T16H, a P450-dependent monooxygenase that catalyses the C-16 hydroxylation of tabersonine, has been shown to be localized in the ER. An *S*-adenosyl-L-methionine-dependent *N*-methyltransferase (NMT), involved in the third-to-last step of vindoline biosynthesis was associated with thylakoid membranes. D4H and DAT, the last two enzymes of vindoline biosynthesis, are localized in the cytosol. The coupling of vindoline with catharanthine to form the bisindole alkaloid anhydrovinblastine occurs through the action of a peroxidase-like enzyme in the vacuole (7). Clearly, at the subcellular level, as is the case at the tissue level, there is a need for significant transport of pathway intermediates among compartments.

The transport of biosynthetic intermediates or final products of alkaloid metabolic pathways among cell types and organelles implies the existence of specific mechanisms for crossing different biological membranes. Relatively little detailed information is available on the transport of alkaloids (4). The experimental data on the transport of alkaloids points to the existence of energy-dependent specific carriers, possibly involving a proton-antiport carrier system (47). The involvement of a directly energized multidrug-resistance protein type ABC (ATP-binding cassette) transporter has recently been demonstrated in the movement of the isoquinoline alkaloid berberine across the plasma membrane of *Coptis japonica* (48). The transport of the same alkaloid across the tonoplast was shown to be mediated by a H^+/berberine antiporter; the involvement of a V-ATPase and a V-PPase in the creation of a proton gradient between the cytosol and the vacuole was also proposed (49). The characterization of transport systems involved in MIA localization will be an important step towards the development of plants engineered for better alkaloid yields.

Factors controlling MIA accumulation in whole plants and untransformed organs as a means for improving yields and understanding *in planta* functions The responsiveness of MIA metabolism to environmental signals and to ontogenetic control has been well established in several plant species. Considering the complex spatial and temporal regulation of alkaloid accumulation previously de-

scribed, it is reasonable to assume that the appropriate management of alkaloid-producing plants can significantly increase bioactive product yields at a relatively low cost of production. Moreover, such treatments for inducing alkaloid accumulation may be applied to engineered plants to further enhance production. In some cases, the content of bioactive MIAs induced by adequate treatment of plants is difficult to surpass even when using the currently available genetic transformation techniques. Therefore, metabolic engineering and physiological manipulation should be used as complementary techniques to improve alkaloid yields.

The identification of environmental and developmental regulatory factors of alkaloid production often occurs in the search for *in planta* functions of these molecules. Evaluating the variation in alkaloid content, biosynthetic enzyme activities and the expression pattern of genes encoding proteins relevant for the metabolic pathway during the main stages of a plant ontogeny, in different organs, and upon exposure to a variety of stress signalling molecules or physical factors may provide important insights into the control and operation of MIA metabolic pathways. The standard factors to be evaluated include wounding, jasmonate treatment, salicylic acid treatment, UV exposure, osmotic/drought stress, temperature variation, light intensity and nutrient stress. The effects of treatment with conventional phytohormones (such as auxins, cytokinins, abscisic acid, ethylene, and gibberellins) are often evaluated as modulating factors of MIA metabolism (50). More recently, other important signalling molecules have come into play, such as nitric oxide (51), and alternative forms of mechanical stress, such as treatment using an ultrasound for *in vitro* cultures, for example, have been employed with the aim of improving the yields of taxol, a diterpene amide (52).

In light of the often insufficient and unstable production of *C. roseus* alkaloids in undifferentiated cultures (*i.e.*, callus and cell cultures), and the potential technical difficulties for treating whole plants on a large scale in the field, a hybrid system for alkaloid production was recently proposed, combining the features of controlled *in vitro* cultures with the use of fully differentiated leaves (53). In this system, surface sterilized leaves are directly cultivated in hormone-free culture medium, with adjusted osmotic pressure, low light irradiation and glucose feeding after 10 d of culture; using this procedure, the concentrations of ajmalicine and serpentine were increased 10-fold above that of the control.

A number of stress and ontogenetic factors have been examined in relation to camptothecin production by *C. acuminata* trees and differentiated cultures. Higher concentrations of camptothecin were associated with younger leaves and branch portions, and a steady decrease in camptothecin concentration was observed during the course of the growing season (54). Progressive drought stress applied to seedlings of *C. acuminata* induced camptothecin accumulation, but the maintenance of the inductive effect was poor and varied among seed sources from different geographic locations (55). The effects of benzyladenine and naphthaleneacetic acid on camptothecin yield in seedlings were also examined. Cytokinin had no effect on yield and auxin decreased the alkaloid concentration (56). Neither treating seedlings with acetylsalicylic acid nor cultivating them in nitrogen enriched hydroponic solutions was able to significantly improve alkaloid yield (57, 58).

In recent years, a chemical survey of the Brazilian species of *Psychotria*, motivated by a combination of ethnobotanical and chemotaxonomical leads, has resulted in the discovery of a number of new MIAs (Fig. 2), several of which presenting relevant biological activities (59, 60) and novel putative biosyntheses (9, 61). These alkaloids show relatively

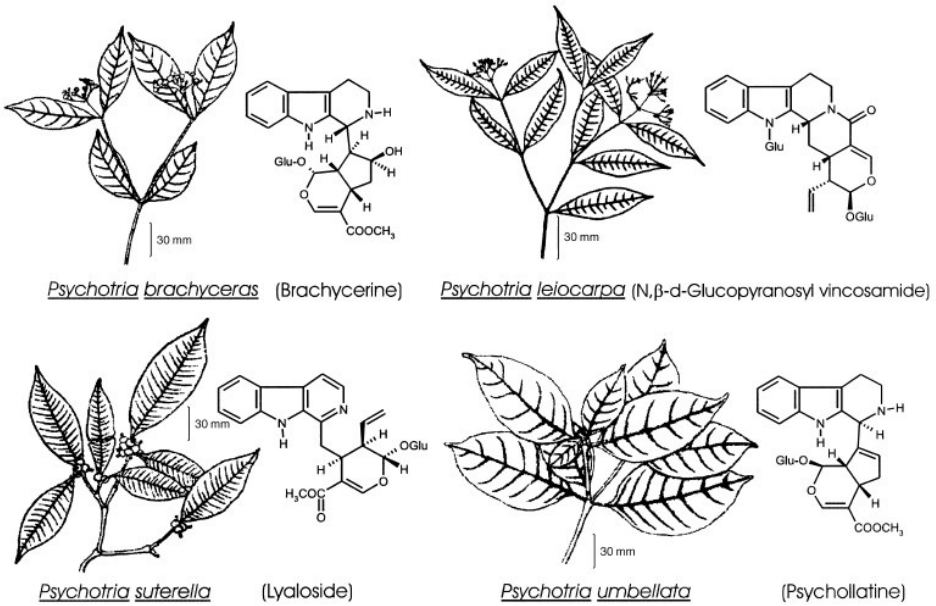


FIG. 2. Examples of monoterpene indole alkaloids of *Psychotria* species from Southern Brazil. For details, see text.

primitive structures compared with the complex bisindolic alkaloids of *C. roseus*. Moreover, *Psychotria* alkaloids retain residues of glucose, which is also distinct from those of vinca. The maintenance of glucose residues is an interesting feature for improved solubility in biological systems and pharmacological applications. Among some of the better-characterized alkaloids from *Psychotria* of Southern Brazil, two display novel biosynthetic features, characterizing a new class of MIAs (brachycerine and psychollatine), because they are produced from tryptamine and a nonsecologanin terpenoid moiety (Fig. 2). Brachycerine from *Psychotria brachyceras* is likely derived from a nonsecologanin terpenoid moiety related to loganin. Psychollatine from *Psychotria umbellata* is presumably derived from geniposide aldehyde. Therefore, STR is not expected to be present in these two species, although some form of STR-like enzyme capable of recognizing the unconventional terpene moieties and condensing them to tryptamine must be present.

A number of relevant activities have been demonstrated for these alkaloids and apparently other properties are still to be unveiled. Brachycerine has been shown to have antioxidant activity (59) and an anti-inflammatory effect in a chemotaxis assay (A.T. Henriques, UFRGS, personal communication). Psychollatine (formerly known as umbellatine) has been shown to have anxiolytic effects by modulating serotonergic $5HTA_{A/C}$ receptors, and opioid-like analgesic effects (60). Recently, an isomer of psychollatine of unknown biological properties (croceaine A) has been isolated from *Palicourea crocea* (62). The affinity of various alkaloids from American *Psychotria* species with those of *Palicourea* has been in agreement with detailed morphological and molecular taxonomy studies suggesting the fusion of both genera in the genus *Heteropsychotria* (9). Lyaloside, the major alkaloid of *Psychotria suterella*, has shown toxicity to and induced convulsions in rodents (63). The main alkaloid of *P. leiocarpa*, *N*,β-D-glucopyranosyl vincosamide (GPV), was the first *N*-glycosylated MIA described (45); leaf aqueous extracts from this plant have shown phytotoxicity against a number of target plant species in both inert and dynamic substrates (Correa and Fett-Neto, unpublished results).

Detailed and comparative studies on the accumulation dynamics of these various chemical entities in intact plants, rooted and unrooted cuttings, root cultures, cell suspensions and embryogenic calli showed a diverse mode of metabolic regulation of MIAs. However, some general features were seen for most of the main *Psychotria* alkaloids examined in detail, such as their absence in roots and undifferentiated callus cultures, presence in differentiated aseptic cultures, relatively high accumulation in reproductive tissues, and regulation by light. The accumulation of alkaloids in differentiated aseptic cultures indicates that these alkaloids are true plant metabolites, not resulting from endophyte associated microorganisms.

Brachycerine is highly responsive to environmental stimuli and also displays developmental control. In a population of adult plants, the highest concentrations of brachycerine were found in inflorescences (0.3% dw), followed by vegetative aerial parts (leaves and green stems), which had concentrations ranging from 0.1% to 0.2% dw. After fruit setting, a significant decrease in brachycerine content was observed in mature fruit and seeds. During seedling development, an increase in leaf content from 0.02% to 0.1% dw was observed between the stages of 2 and 14 leaves. The seasonal pattern of alkaloid accumulation in leaves indicated lower contents in summer, although in the second year, with higher and better distributed rain levels, leaf contents remained stable and lower, suggesting an induction of production by drought stress (8).

The higher accumulation of brachycerine in inflorescences and leaves suggested a protective role of the alkaloid in *P. brachyceras*. In fact, brachycerine is locally induced by leaf wounding within 24 h and by jasmonate treatment of undamaged plants, suggesting the participation of the octadecanoid pathway in the signalling of brachycerine induction (8). It would be interesting to look for *orca*-like genes in the *Psychotria* genome. Salicylic acid was unable to induce brachycerine accumulation (8).

The most effective induction of brachycerine accumulation was in response to UV irradiation (59). The UV-response pathway has similarities with the wound-response pathway (64), and UV irradiation has been shown to induce the expression of *tdc* in *C. roseus* mediated by the transcription factor GT-1 (65). In *P. brachyceras* cuttings, a 10-fold increase in brachycerine content was observed upon irradiation with UV-C light for 16 h a day after 6 d, whereas 4 h a day of UV-C or 16 h a day of UV-B caused a 2-fold increase within 4 to 6 d. Because brachycerine has a peak absorbance at 223 nm, it could be acting as a filter against this harmful environmental factor. Moreover, antioxidant assays showed that brachycerine was also capable of quenching singlet oxygen, indicating a possible protective role against the secondary effects of UV irradiation on the cytosol. Therefore, UV irradiation can be an effective means to quickly enhance the yields of brachycerine. Greening and/or UV irradiation of callus or rhizogenic callus cultures of *P. brachyceras* did not induce the accumulation of brachycerine, indicating the importance of differentiated shoots for its production. The fact that the induction of brachycerine accumulation upon UV irradiation was also observed in unrooted cuttings indicates that its biosynthesis occurs in shoots (59).

The production of psychollatine, which is structurally related to brachycerine, is a lot less responsive to environmental stimuli. Leaves, young inflorescences and fruit pulp contained 2.5% to 4.5% dw of psychollatine, whereas seeds and roots had amounts of 0.2% and 0.05%, respectively. Psychollatine accumulation was not seasonally dependent and leaf content was unchanged by wounding, UV irradiation, salicylic acid or hydrogen peroxide treatment, but decreased by exposure to auxin. The harvested leaf content decreased at 65°C, but was otherwise stable (Paranhos and Fett-Neto, unpublished results). Interestingly, the basal contents of psychollatine are comparable to the maximum induction contents of brachycerine (upon UV-C treatment), characterizing a phytoanticipin-like type of alkaloid accumulation profile in psychollatine and a phytoalexin-like profile in brachycerine (8).

A somatic embryo regeneration protocol of *P. umbellata* from rhizogenic calli was developed wherein regenerated plants could accumulate psychollatine in amounts equiva-

lent to those of field-grown plants. Nevertheless, no psychollatine was detected in calli or rhizogenic calli; however, alkaloid biosynthetic capacity was recovered upon shoot differentiation (66). These results are similar to those described for brachycerine. It will be interesting to investigate the expression profile of genes encoding alkaloid biosynthetic enzymes in *P. umbellata* and *P. brachyceras*, as well as *orca* homologs to examine differences in the regulation of distinct alkaloid production strategies observed in these related species. For both *P. brachyceras* and *P. umbellata*, a gradient of variability in alkaloid yield among different individuals across the seasons in two subsequent years clearly indicated that a genetic component is involved in metabolite yield; this is obviously a factor to explore to obtain elite germplasm for both physiological manipulation and metabolic engineering, not only for these, but also for other MIA-producing species. Similar approaches have been used for *C. roseus* (26).

The alkaloid GPV of *P. leiocarpa* displays a clear temporal and spatial regulation of its accumulation. GPV was also restricted to shoots, and occurred mainly in leaves, but was absent in roots. GPV content increased with the age of seedlings, as in the case of brachycerine in *P. brachyceras*. GPV accumulation was strongly induced by light, increasing with photomorphogenesis, independently of the presence of carbohydrates; this indicates that the carbon nutrition-related role of light is not involved in this induction (45). Exogenous carbohydrates repressed GPV accumulation, presumably by inhibiting the expression of genes encoding the plastid-located enzymes involved in secologanin biosynthesis, the terpenoid moiety in GPV.

CONCLUSION

The first study of the regeneration of *C. roseus* plants from *Agrobacterium*-transformed tissues has recently been reported (67). Wild-type *A. rhizogenes* was used in this study and no gene of interest was therefore transferred. Much earlier, the regeneration of *R. serpentina* transgenic plants employing essentially the same approach has been reported (68). Hence, the combination of techniques for regenerating plants from hairy roots and the transgenic expression of specific MIA-synthesizing or -controlling genes under the regulation of inducible promoters in alkaloid-producing plants such as *C. roseus* seems to be the most promising strategy for the future and may prove the validity of genetic transformation to enhance the productivity of MIAs in whole plants as well as in *in vitro* cell and tissue cultures. The complex spatial and temporal regulations of alkaloid metabolism, often dependent on highly differentiated tissues or cells, and its close relationship with environmental stimuli have to be fully characterized to further improve alkaloid yield both in wild type and transgenic plants. The elucidation of *in planta* roles for alkaloids may also contribute to this end. The complementary approaches of appropriate metabolic engineering and applied physiological treatments for the induction of alkaloid accumulation hold the key for better and sustained production systems of bioactive MIAs, and should be tested on various species of interest. Considering that a large proportion of the world's phytodiversity has not been chemically and pharmacologically described, particularly in the tropics and subtropics, the reservoir of new alkaloid molecules, biosynthetic enzymes and regulatory proteins is sure to continue expanding in the near future. For bioscientists and bioengineers, the possibilities to rationally explore these valuable resources are literally endless.

ACKNOWLEDGMENTS

We apologize to colleagues whose studies could not be cited due to space constraints. Research in the authors' laboratories was supported by grants and bursaries from the Brazilian agencies CNPq, CAPES, and FAPERGS.

REFERENCES

1. **van der Heijden, R., Jacobs, D. I., Snoeijer, W., Hallard, D., and Verpoorte, R.:** The *Catharanthus* alkaloids: pharmacognosy and biotechnology. Curr. Med. Chem., **11**, 1241–1253 (2004).
2. **Sudha, C. G., Obul Reddy, B., Ravishankar, G. A., and Seeni, S.:** Production of ajmalicine and ajmaline in hairy root cultures of *Rauvolfia micrantha* Hook f., a rare and endemic medicinal plant. Biotechnol. Lett., **25**, 631–636 (2003).
3. **Hughes, E. H. and Shanks, J. V.:** Metabolic engineering of plants for alkaloid production. Metab. Eng., **4**, 41–48 (2002).
4. **Hashimoto, T. and Yamada, Y.:** New genes in alkaloid metabolism and transport. Curr. Opin. Biotechnol., **14**, 163–168 (2003).
5. **Gantet, P. and Memelink, J.:** Transcription factors: tools to engineer the production of pharmacologically active plant metabolites. Trends Pharmacol. Sci., **23**, 563–569 (2002).
6. **Jacobs, D. I., Gaspari, M., van der Greef, J., van der Heijden, R., and Verpoorte, R.:** Proteome analysis of the medicinal plant *Catharanthus roseus*. Planta, **221**, 690–704 (2005).
7. **Facchini, P. J.:** Alkaloid biosynthesis in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol., **52**, 29–66 (2001).
8. **Gregianini, T. S., Porto, D. D., Nascimento, N. C., Fett, J. P., Henriques, A. T., and Fett-Neto, A. G.:** Environmental and ontogenetic control of accumulation of brachycerine, a bioactive indole alkaloid from *Psychotria brachyceras*. J. Chem. Ecol., **30**, 2023–2036 (2004).
9. **Lopes, S. O., Von Poser, G. L., Kerber, V. A., Farias, F. M., Konrath, E. L., Moreno, P. R. H., Sobral, M., Zuanazzi, J. A., and Henriques, A. T.:** Taxonomic significance of alkaloids and iridoid glucosides in the tribe Psychotrieae (Rubiaceae). Biochem. Syst. Ecol., **32**, 1187–1195 (2004).
10. **Shanks, J. V. and Morgan, J.:** Plant "hairy root" culture. Curr. Opin. Biotechnol., **10**, 151–155 (1999).
11. **Giri, A. and Lakshmi Narasu, M.:** Transgenic hairy roots: recent trends and applications. Biotechnol. Adv., **18**, 1–22 (2000).
12. **Sevon, N. and Oksman-Caldentey, K. M.:** *Agrobacterium rhizogenes*-mediated transformation: root cultures as a source of alkaloids. Planta Med., **68**, 859–868 (2002).
13. **Batra, J., Dutta, A., Singh, D., Kumar, S., and Sen, J.:** Growth and terpenoid indole alkaloid production in *Catharanthus roseus* hairy root clones in relation to left- and right-termini-linked Ri T-DNA gene integration. Plant Cell Rep., **23**, 148–154 (2004).
14. **Lorence, A. and Nessler, C. L.:** Camptothecin, over four decades of surprising findings. Phytochemistry, **65**, 2735–2749 (2004).
15. **Saito, K., Sudo, H., Yamazaki, M., Koseki-Nakamura, M., Kitajima, M., Takayama, H., and Aimi, N.:** Feasible pro-

duction of camptothecin by hairy root culture of *Ophiorrhiza pumilla*. Plant Cell Rep., **20**, 267–271 (2001).
16. **Berlin, J. and Fecker, L.:** Metabolic engineering of enzymes diverting amino acids into secondary metabolism, p. 195. *In* Verpoorte, R. and Alfermann, A. W. (ed.), Metabolic engineering of plant secondary metabolism. Agritech, Shrub Oak, New York (2000).
17. **Verpoorte, R., van der Heijden, R., and Memelink, J.:** Engineering the plant cell factory for secondary metabolite production. Transgenic Res., **9**, 323–343 (2000).
18. **Verpoorte, R. and Memelink, J.:** Engineering secondary metabolite production in plants. Curr. Opin. Biotechnol., **13**, 181–187 (2002).
19. **Hughes, E. H., Hong, S. B., Gibson, S. I., Shanks, J. V., and San, K. Y.:** Expression of a feedback-resistant anthranilate synthase in *Catharanthus roseus* hairy roots provides evidence for tight regulation of terpenoid indole alkaloid levels. Biotechnol. Bioeng., **86**, 718–727 (2004).
20. **Goddijn, O. J. M., Pennings, E. J. M., van der Helm, P., Schilperoort, R. A., Verpoorte, R., and Hoge, J. H. C.:** Overexpression of a tryptophan decarboxylase cDNA in *Catharanthus roseus* crown gall calluses results in increased tryptamine levels but not in increased terpenoid indole alkaloid production. Transgenic Res., **4**, 315–323 (1995).
21. **Canel, C., Lopes-Cardoso, I., Whitmer, S., van der Fits, L., Pasquali, G., van der Heijden, R., Hoge, J. H. C., and Verpoorte, R.:** Effects of over-expression of strictosidine synthase and tryptophan decarboxylase on alkaloid production by cell cultures of *Catharanthus roseus*. Planta, **205**, 414–419 (1998).
22. **Whitmer, S., Canel, C., van der Heijden, R., and Verpoorte, R.:** Long-term instability of alkaloid production by stably transformed cell lines of *Catharanthus roseus*. Plant Cell Tissue Org. Cult., **74**, 73–80 (2003).
23. **Berlin, J., Rugenhagen, C., Dietze, P., Fecker, L. F., Goddijn, O. G. M., and Hoge, J. H. C.:** Increased production of serotonin by suspension and root cultures of *Peganum harmala* transformed with a tryptophan decarboxylase cDNA clone from *Catharanthus roseus*. Transgenic Res., **2**, 336–344 (1993).
24. **Geerlings, A., Hallard, D., Martinez Caballero, A., Lopes Cardoso, I., van der Heijden, R., and Verpoorte, R.:** Alkaloid production by a *Cinchona officinalis* 'Ledgeriana' hairy root culture containing constitutive expression constructs of tryptophan decarboxylase and strictosidine synthase cDNAs from *Catharanthus roseus*. Plant Cell Rep., **19**, 191–196 (1999).
25. **Hughes, E. H., Hong, S-B., Gibson, S. I., Shanks, J. V., and Sana, K-Y.:** Metabolic engineering of the indole pathway in *Catharanthus roseus* hairy roots and increased accumulation of tryptamine and serpentine. Metab. Eng., **6**, 268–276 (2004).
26. **Dutta, A., Batra, J., Pandey-Rai, S., Singh, D., Kumar, S., and Sen, J.:** Expression of terpenoid indole alkaloid corresponds to accumulation of related alkaloids in *Catharanthus roseus* (L.) G. Don. Planta, **220**, 376–383 (2005).
27. **Di Fiore, S., Li, Q., Leech, M. J., Schuster, F., Emans, N., Fischer, R., and Schillberg, S.:** Targeting tryptophan decarboxylase to selected subcellular compartments of tobacco plants affects enzyme stability and *in vivo* function and leads to a lesion-mimic phenotype. Plant Physiol., **129**, 1160–1169 (2002).
28. **Li, Q. R., Di Fiore, S., Fischer, R., and Wang, M.:** Expression of tryptophan decarboxylase in chloroplasts of transgenic tobacco plants. Bot. Bull. Acad. Sin., **44**, 193–198 (2003).
29. **Aoyagi, H., Sakamoto, Y., Asada, M., and Tanaka, H.:** Indole alkaloids production by *Catharanthus roseus* protoplasts with artificial cell walls containing of gucuronic acid rich alginate gel. J. Ferment. Bioeng., **85**, 306–311 (1998).
30. **Hong, S-B., Peebles, C. A. M., Shanks, J. V., San, K-Y., and Gibson, S. I.:** Expression of the *Arabidopsis* feedback-insensitive anthranilate synthase holoenzyme and tryptophan decarboxylase genes in *Catharanthus roseus* hairy roots. J. Biotechnol., **122**, 28–38 (2006).
31. **Laflamme, P., St-Pierre, B., and De Luca, V.:** Molecular and biochemical analysis of a Madagascar periwinkle root-specific minovincinine-19-hydroxy-*O*-acetyltransferase. Plant Physiol., **125**, 189–198 (2001).
32. **Pan, X-W., Shi, Y-Y., Liu, X., Gao, X., and Lu, Y-T.:** Influence of inorganic microelements on the production of camptothecin with suspension cultures of *Camptotheca acuminata*. Plant Growth Regul., **44**, 59–63 (2004).
33. **Pan, X-W., Xu, H-H., Liu, X., Gao, X., and Lu, Y-T.:** Improvement of growth and camptothecin yield by altering nitrogen source supply in cell suspension cultures of *Camptotheca acuminata*. Biotechnol. Lett., **26**, 1745–1748 (2004).
34. **Oksman-Caldentey, K. M. and Inzé, D.:** Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. Trends Plant Sci., **9**, 433–440 (2004).
35. **Vom Endt, D., Kijne, J. W., and Memelink, J.:** Transcription factors controlling plant secondary metabolism: what regulates the regulators? Phytochemistry, **61**, 107–114 (2002).
36. **Pauw, B., Hilliou, F. A. O., Martin, V. S., Chatel, G., Wolf, C. J. F., Champion, A., Pré, M., van Duijn, B., Kijne, J. W., van der Fits, L., and Memelink, J.:** Zinc finger proteins act as transcriptional repressors of alkaloid biosynthesis genes in *Catharanthus roseus*. J. Biol. Chem., **279**, 52940–52948 (2004).
37. **Facchini, P. J., Bird, D. A., and St. Pierre, B.:** Can *Arabidopsis* make complex alkaloids? Trends Plant Sci., **9**, 116–122 (2004).
38. **St-Pierre, B., Vazquez-Flota, F. A., and De Luca, V.:** Multicellular compartmentation of *Catharanthus roseus* alkaloid biosynthesis predicts intercellular translocation of a pathway intermediate. Plant Cell, **11**, 887–900 (1999).
39. **Irmler, S., Schroder, G., St-Pierre, B., Crouch, N., Hotze, M., Schmidt, J., Strack, D., Matern, U., and Schroeder, J.:** Indole alkaloid biosynthesis in *Catharanthus roseus*: new enzyme activities and identification of cytochrome P450 CYP72A1 as secologanin synthase. Plant J., **24**, 797–804 (2000).
40. **Burlat, V., Oudin, A., Courtois, M., Rideau, M., and St-Pierre, B.:** Co-expression of three MEP pathway genes and geraniol-10-hydroxylase in internal phloem parenchyma of *Catharanthus roseus* implicates multicellular translocation of intermediates during the biosynthesis of monoterpene indole alkaloids and isoprenoid-derived primary metabolites. Plant J., **38**, 131–141 (2004).
41. **Contin, A., Van der Heijden, R., Lefeber, A. W. M., and Verpoorte, R.:** The iridoid glucoside secologanin is derived from the novel triose phosphate/pyrivate pathway in *Catharanthus roseus*. FEBS Lett., **434**, 413–416 (1998).
42. **De Luca, V. and St. Pierre, B.:** The cell and developmental biology of alkaloid biosynthesis. Trends Plant Sci., **5**, 168–173 (2000).
43. **Bird, D. A., Franceschi, V. R., and Facchini, P. J.:** A tale of three cell types: alkaloid biosynthesis is localized to sieve elements in opium poppy. Plant Cell, **15**, 2626–2635 (2003).
44. **Samanani, N., Park, S-U., and Facchini, P. J.:** Cell type-specific localization of transcripts encoding nine consecutive enzymes involved in protoberberine alkaloid biosynthesis. Plant Cell, **17**, 915–926 (2005).
45. **Henriques, A. T., Lopes, S. O., Paranhos, J. T., Gregianini, T. S., Von Poser, G. L., Fett-Neto, A. G., and Schripsema, J.:** *N*,β-D-Glucopyranosyl vincosamide, a light regulated indole alkaloid from the shoots of *Psychotria leiocarpa*. Phytochemistry, **65**, 449–454 (2004).

46. **Schuhr, C. A., Radykewicz, T., Sagner, S., Latzel, C., Zenk, M., Arigoni, D., Bacher, A., Rohdich, F., and Eisenreich, W.:** Quantitative assessment of crosstalk between the two isoprenoid biosynthesis pathways in plants by NMR spectroscopy. Phytochem. Rev., **2**, 3–16 (2003).
47. **Mende, P. and Wink, M.:** Uptake of the quinolizidine alkaloid lupanine by protoplasts and isolated vacuoles of suspension-cultured *Lupinus polyphyllus* cells. Diffusion or carrier mediated transport? J. Plant Physiol., **129**, 229–242 (1987).
48. **Shitan, N., Bazin, I., Dan, K., Obata, K., Kigawa, K., Ueda, K., Sato, F., Forestier, C., and Yazaki, K.:** Involvement of CjMDR1, a plant multidrug-resistance type ATP-binding cassette protein, in alkaloid transport in *Coptis japonica*. Proc. Natl. Acad. Sci. USA, **100**, 751–756 (2003).
49. **Otani, M., Shitan, N., Sakai, K., Martinoia, E., Sato, F., and Yazaki, K.:** Characterization of vacuolar transport of the endogenous alkaloid berberine in *Coptis japonica*. Plant Physiol., **138**, 1939–1946 (2005).
50. **El-Sayed, M. and Verpoorte, R.:** Effect of phytohormones on growth and alkaloid accumulation by a *Catharanthus roseus* cell suspension cultures fed with alkaloid precursors tryptamine and loganin. Plant Cell Tissue Org. Cult., **68**, 265–270 (2002).
51. **Xu, M. and Dong, J.:** Nitric oxide stimulates indole alkaloid production in *Catharanthus roseus* cell suspension cultures through a protein kinase-dependent signal pathway. Enzyme Microb. Technol., **37**, 49–53 (2005).
52. **Wu, J. and Lin, L.:** Enhancement of taxol production and release in *Taxus chinensis* cell cultures by ultrasound, methyl jasmonate and *in situ* solvent extraction. Appl. Microb. Biotechnol., **62**, 151–155 (2003).
53. **Iwase, A., Aoyagi, H., Ohme-Takagi, M., and Tanaka, H.:** Development of a novel system for producing ajmalicine and serpentine using direct culture of leaves in *Catharanthus roseus*. J. Biosci. Bioeng., **99**, 208–215 (2005).
54. **Liu, Z., Carpenter, S. B., Bourgeois, W. J., Yu, Y., Constantin, R. J., Falcon, M. J., and Adams, J. C.:** Variations in the secondary metabolite camptothecin in relation to tissue age and season in *Camptotheca acuminata*. Tree Physiol., **18**, 265–270 (1998).
55. **Liu, Z.:** Drought-induced *in vivo* synthesis of camptothecin in *Camptotheca acuminata* seedlings. Physiol. Plant., **110**, 483–488 (2000).
56. **Li, Z. and Liu, Z.:** Effects of benzyladenine and naphthalene acetic acid on growth and camptothecin accumulation in *Camptotheca acuminata* seedlings. J. Plant Growth Regul., **22**, 205–216 (2003).
57. **Li, Z. and Liu, Z.:** Camptothecin accumulation in *Camptotheca acuminata* seedlings in response to acetylsalicylic acid treatment. Can J. Plant Sci., **84**, 885–889 (2004).
58. **Li, Z. and Liu, Z.:** Camptothecin production in *Camptotheca acuminata* cultured hydroponically and with nitrogen enrichments. Can J. Plant Sci., **85**, 447–452 (2004).
59. **Gregianini, T. S., Silveira, V. C., Porto, D. D., Kerber, V. A., Henriques, A. T., and Fett-Neto, A. G.:** The alkaloid brachycerine is induced by ultraviolet radiation and is a singlet oxygen quencher. Photochem. Photobiol., **78**, 470–474 (2003).
60. **Both, F. L., Meneghini, L., Kerber, V. A., Henriques, A. T., and Elisabetsky, E.:** Psychopharmacological profile of the alkaloid psychollatine as a $5HT2_{A/C}$ serotonin modulator. J. Nat. Prod., **68**, 374–380 (2005).
61. **Kerber, V. A., Gregianini, T. S., Paranhos, J. T., Schwambach, J., Farias, F., Fett, J. P., Fett-Neto, A. G., Zuanazzi, J. A., Quirion, J. C., Elisabetsky, E., and Henriques, A. T.:** Brachycerine, a novel monoterpene indole alkaloid from *Psychotria brachyceras*. J. Nat. Prod., **64**, 677–679 (2001). Erratum: J. Nat. Prod., **66**, 1038–1039 (2003).
62. **Düsman, L. T., Jorge, T. C. M., Souza, M. C., Eberlin, M. N., Meurer, E. C., Bocca, C. C., Basso, E. A., and Sarragiotto, M. H.:** Monoterpene indole alkaloids from *Palicourea crocea*. J. Nat. Prod., **67**, 1886–1888 (2004).
63. **Van De Santos, L., Fett-Neto, A. G., Kerber, V. A., Elisabetsky, E., Quirion, J. C., and Henriques, A. T.:** Indole monoterpene alkaloids from leaves of *Psychotria suterella* Müll. Arg. (Rubiaceae). Biochem. Syst. Ecol., **29**, 1185–1187 (2001).
64. **Conconi, A., Smerdon, M. J., Howe, G. A., and Ryan, C. A.:** The octadecanoid signaling pathway in plants mediates a response to ultraviolet radiation. Nature, **383**, 826–829 (1996).
65. **Ouwerkerk, P. B. F., Trimborn, T. O., Hiliou, F., and Memelink, J.:** Nuclear factors GT-1 and 3AF1 interact with multiple sequences within the promoter of the *tdc* gene of Madagascar periwinkle: GT-1 is involved in UV light-induced expression. Mol. Gen. Genet., **261**, 610–622 (1999).
66. **Paranhos, J. T., Fragoso, V., Henriques, A. T., Ferreira, A. G., and Fett-Neto, A. G.:** Regeneration of *Psychotria umbellata* and production of the analgesic indole alkaloid umbellatine. Tree Physiol., **25**, 251–255 (2005).
67. **Choi, P. S., Kim, Y. D., Choi, K. M., Chung, H. J., Choi, D. W., and Liu, J. R.:** Plant regeneration from hairy-root cultures transformed by infection with *Agrobacterium rhizogenes* in *Catharanthus roseus*. Plant Cell Rep., **22**, 828–831 (2004).
68. **Benjamin, B. D., Roja, G., and Heble, M. R.:** *Agrobacterium rhizogenes*-mediated transformation of *Rauvolfia serpentina*: regeneration and alkaloid synthesis. Plant Cell Tissue Org. Cult., **35**, 253–257 (1993).