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Ciclotídeos e alcalóides de *Psychotria* spp. do Sul do Brasil: características e possíveis funções

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Tese

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Keep your mind open (but not so open that your brains fall out),
and make everything as simple as possible, but not simpler
(W. Kotschnig; A. Einstein)

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Resumo

Alcalóides estão entre os principais compostos de defesa contra herbívoros presentes em plantas. Apresentam diversas atividades biológicas, incluindo atividade anticâncer, observada, por exemplo, em alcalóides indólicos de *Catharanthus roseus*. A família Rubiaceae abriga importantes espécies produtoras de alcalóides, tais como o cafeeiro (*Coffea arabica*) e a quina (*Cinchona officinalis*), assim como plantas produtoras de peptídeos circulares (ciclotídeos), caso da kalata-kalata (*Oldenlandia affinis*). *Psychotria* é o maior gênero de Rubiaceae, sendo rico em espécies acumuladoras de alcalóides. A presença de plantas produtoras de ciclotídeos também foi relatada neste gênero. Algumas espécies de *Psychotria* do Sul do Brasil são grandes produtoras de alcalóides indólicos, que alcançam até 4% do peso seco das folhas. A elucidação de fatores afetando o metabolismo de alcalóides e de ciclotídeos pode auxiliar na melhor compreensão de sua função ecológica, além de resultar em possíveis avanços no aumento de produtividade para aplicações destas moléculas. Neste trabalho, o controle espaço-temporal do acúmulo do alcalóide indólico antioxidante N,*β*-D-glicopiranosil vincosamida (GPV) em plântulas de *Psychotria leiocarpa* foi estudado em relação à irradiância e acúmulo órgão-específico vegetativo e reprodutivo. Luz, particularmente quando enriquecida na faixa do vermelho-extremo e azul, promoveu o acúmulo do alcalóide. Maiores concentrações de GPV foram observadas em botões florais e flores. GPV mostrou-se um eficiente antioxidante contra oxigênio singleto e superóxido em relação a alcalóides correlatos, sendo comparável à rutina e Trolox^{TM}. Espécies sensíveis a tratamento agudo com UV-B tornaram-se tolerantes após aplicação de GPV em suas superfícies foliares. Os altos teores de GPV em *P. leiocarpa* (2,5% peso seco), em consonância com o observado em algumas espécies congêneres (braquicerina em *P. brachyceras* com até 1,8% peso seco e psicolatina em *P. umbellata* com até 4% peso seco) parecem auxiliar as plantas a mitigar o estresse oxidativo em condições desfavoráveis, sendo, por exemplo, eficientes na proteção de folhas à exposição aguda à UV-B. Porém, estes alcalóides aparentemente não exibem toxidez contra predadores, apesar do baixo grau de dano observado nestas plantas em seu ambiente natural. Por outro lado, *P. brachyceras* e *P. leiocarpa* se mostraram espécies extremamente ricas em ciclotídeos, podendo abrigar mais de 17 destes peptídeos, totalizando teores próximos de 0,2% do peso seco de folhas. A função ecológica proposta na literatura para ciclotídeos envolve proteção contra herbívoros, e o ciclotídeo psyleio A, encontrado nas duas espécies de *Psychotria* estudadas, apresentou eficiente ação inseticida contra *Helicoverpa armigera*. A concentração dos principais ciclotídeos mostrou-se constitutiva frente a diversos tipos de elicitação. As ações combinadas de alcalóides monoterpeno indólicos antioxidantes e ciclotídeos inseticidas parecem constituir uma estratégia eficiente na proteção destas espécies de *Psychotria* contra estresses bióticos e abióticos, os quais frequentemente ocorrem de forma combinada em condições de campo.

Palavras chave: ciclotídeos, inseticida, herbivoria, metabolismo secundário, alcalóides monoterpeno indólicos, antioxidante

Abstract

Alkaloids are among the main defense compounds against herbivores in plants. These metabolites present an array of bioactivities, including anticancer properties, observed for example, in indole alkaloids of *Catharanthus roseus*. Rubiaceae encompasses important alkaloid-accumulating plants, such as coffe (*Coffea arabica*) and quine (*Cinchona officinalis*), as well as plants producing circular peptides, as is the case with kalata-kalata (*Oldenlandia affinis*). *Psychotria* is the largest genus within Rubiaceae, being rich in alkaloid accumulating plants. The presence of cyclotide-producing plants is also reported in this genus. Some South Brazilian *Psychotria* species are extremely rich in indole alkaloids, reaching contents up to 4% dry weight. The elucidation of factors affecting alkaloid and cyclotide metabolism may help to understand ecological function and lead to advances for increasing productivity of these molecules for various aplications. In this work, the spatiotemporal control of concentrations of the antioxidant indole alkaloid N,*β*-D-glucopyranosyl vincosamide (GPV) in seedlings of *Psychotria leiocarpa* was studied in relation to irradiance and organ-specific accumulation in reproductive and vegetative structures. Light, particularly if enriched in the far-red and blue range, promoted alkaloid accumulation. Higher concentrations of GPV were found in flowers and floral buds. GPV was an efficient antioxidant against singlet oxygen and superoxide relative to similar alkaloids, being comparable to rutin and Trolox^{TM}. Plant species sensitive to acute UV-B treatment became tolerant after GPV application on their leaf surfaces. The high content of GPV in *P. leiocarpa* (2.5% dry weight), in line with closely related species (*P. brachyceras* with up to 1.8% dry weigth of brachycerine and *P.* umbellata with up to 4% dry weight of psychollatine) seems to aid these plants in mitigating oxidative stress in unfavorable conditons, for example, being efficient in leaf protection upon exposure to acute doses of UV-B. However, these alkaloids do not show overt toxicity against predators, in spite of the low degree of damage observed in these plants in their natural environment. On the other hand, *P. brachyceras* and *P. leiocarpa* proved to be extremely rich in cyclotides, bearing more than 17 of these peptides, reaching concentrations close to 0.2% dry weight of leaves. The ecological function proposed for cyclotides involves protection against herbivores. Indeed, the cyclotide psyleio A, found in both species investigated showed effective insecticide activity on *Helicoverpa armigera*. The concentration of the main cyclotides was constitutive even after several types of elicitation treatments. The combined actions of antioxidant monoterpene indole alkaloids and insecticidal cyclotides seem to constitute an efficient strategy in the protection of these species of *Psychotria* against biotic and abiotic stresses, which often take place in combined fashion under field conditions.

Key words: cyclotides, insecticide, herbivory, secondary metabolism, monoterpene indole alkaloids, antioxidant

Sumário

Introdução

Em sua história evolutiva, desde o surgimento de seus ancestrais relacionados às algas verdes, há mais de 1,4 bilhão de anos (Hedges *et al.*, 2004), as plantas desenvolveram complexas e distintas estratégias de ocupação do ambiente e de proteção contra estresses ambientais. No período Ordoviciano, há 470 milhões de anos, conquistaram o habitat terrestre (Wellman *et al.*, 2003; Steemans *et al.*, 2009) sendo necessárias adaptações adicionais à perda de água, sustentação, distribuição de nutrientes e mecanismos de reprodução.

Diferentemente de outros seres vivos que podem se locomover para realizar suas atividades, as plantas têm o desafio de, enquanto sésseis, desenvolver-se e propagar seus genes ao mesmo tempo em que enfrentam competidores, predadores e condições ambientais desfavoráveis. Como representantes do primeiro nível trófico da cadeia alimentar, o investimento em proteção contra patógenos e contra predadores é essencial para a sobrevivência, sendo necessário um fino balanço entre alocação de recursos para crescimento *versus* defesa (Meldau *et al.*, 2012). Mecanismos de proteção contra a perda de água e entrada de patógenos estão presentes em vários níveis, sendo a primeira barreira constituída por substâncias hidrofóbicas (suberina e cutina) nas partes mais externas do caule e das folhas (no súber e na cutícula, respectivamente) (Taiz *et al.*, 2015).

Associações ecológicas podem ser eficazes para afastar herbívoros, como o caso de algumas espécies de *Cecropia* que abrigam formigas do gênero *Azteca*, altamente agressivas contra invasores, conferindo uma vantagem à planta durante seu crescimento, afastando herbívoros, além de ser uma fonte adicional de nitrogênio (proveniente de resíduos dos recursos utilizados pelas formigas) (Oliveira *et al.*, 2015). Os mecanismos de proteção contra herbívoros são diversos e incluem a presença de estruturas que dificultam fisicamente o ataque de predadores, entre eles o acúmulo de oxalato de cálcio em partes da planta, que formam cristais em formato de agulhas (ráfides) capazes de perfurar orgãos e tecidos (e.g. Araceae, Aquifoliaceae, Oxalidaceae), a presença de acúleos (e.g. Rosaceae, Bombacaceae), e a presença de espinhos (e.g. Cactaceae, Rutaceae, Euphorbiaceae). Várias outras estratégias envolvem defesas químicas.

Compostos de defesa estão presentes em diversas famílias de plantas e estes podem auxiliar na proteção tanto diretamente (e.g. amargor, inibidores de protease) quanto indiretamente (e.g. antioxidantes) e na interação com outros organismos por intermédio de compostos orgânicos voláteis. Tais metabólitos voláteis, geralmente terpenos ou fenólicos,

podem prevenir ataques futuros estimulando o acúmulo de compostos de defesa em partes de plantas não atacadas antes da chegada do predador (Elizabeth *et al.*, 2014; Maag *et al.*, 2015). Além disso, plantas podem promover a produção de compostos de defesa de modo a atrair inimigos naturais de seus predadores (Stam *et al.*, 2014) ou, ainda, para modular a ação de polinizadores (Lucas-Barbosa 2016).

A percepção antecipada de ataques futuros pode conferir uma vantagem. Recentemente foi descoberto que plantas são capazes de perceber sons específicos, sendo o som gerado durante a mastigação das folhas pelas lagartas um eficiente sinal para induzir compostos de defesa (ensaio realizado com *Arabidopsis*) (Appel e Cocroft, 2014). Fezes de lagartas também podem induzir genes de defesa em resposta a dano mecânico, bem como genes relacionados a patógenos (ensaio realizado com *Zea mays*) (Ray *et al.*, 2015).

Além de diversos compostos e estratagemas selecionados pelas pressões evolutivas, novas formas de defesa e novas funções de antigos compostos podem surgir em diferentes ambientes. Determinadas características podem conferir uma nova adaptação biológica, mesmo não sendo o resultado de pressões seletivas anteriores, sendo esse fenômeno denominado pré-adaptação ou exaptação (Gould & Vrba, 1982). Foi demonstrado *in silico* que sistemas metabólicos possuem um grande potencial para inovações evolutivas de origem não adaptativa (exaptações) (Barve e Wagner, 2013) o que implica, tomando-se os devidos cuidados, que alguns metabólitos podem adquirir mais de uma função, incluindo uma função de origem não adaptativa. A cafeína, um alcalóide do grupo das xantinas, por exemplo, ocorre naturalmente em folhas e frutos de algumas plantas (e.g. *Coffea*, *Camellia*) e, devido a seu amargor, confere proteção à planta repelindo predadores. Interessantemente, quando presente no néctar em quantidades moderadas, a cafeína aumenta drasticamente a atração de abelhas, por meio de um efeito de "memória de recompensa", oferecendo uma função adicional, completamente oposta à original que, porém, interfere diretamente de forma positiva na reprodução da planta (Couvillon *et al.*, 2015).

1.1. Metabolismo vegetal

Classicamente, o metabolismo das plantas é dividido em primário e secundário para facilitar a compreensão e comunicação acerca do tema. Esta, no entanto, é uma separação arbitrária, uma vez que ambos são essenciais para a planta crescendo no ambiente natural (Firn & Jones, 2009) e estão fortemente imbricados nas vias metabólicas. A separação dos dois tipos de metabolismo é por vezes tênue, como é o caso da lignina, que deriva da rota biossintética dos compostos fenólicos, possui uma função primária estrutural, mas também é fortemente responsiva a estímulos ambientais, com participação em respostas de defesa (Moura *et al.*, 2010). Diversas proteínas que atuam como compostos de defesa também são exemplos de interface entre o metabolismo primário e secundário.

O metabolismo primário relaciona-se diretamente ao crescimento, desenvolvimento e reprodução das plantas, atuando em processos como fotossíntese, respiração, transporte de solutos, translocação, síntese proteica, assimilação de nutrientes, diferenciação celular e a formação de metabólitos primários (carboidratos, proteínas, lipídeos e ácidos nucleicos) (Taiz *et al.*, 2015). Metabolismo secundário é aquele não relacionado diretamente aos processos citados anteriormente e que geralmente não está presente em todo reino vegetal, sendo restrito a algumas famílias botânicas. O metabolismo secundário tem uma importante função ecoquímica, na interação das plantas com o ambiente. Devido à alocação de recursos essenciais como carbono e nitrogênio, a manutenção do metabolismo secundário é regida por pressões seletivas onde a indução de defesas ocorre preferencialmente em situações energeticamente favoráveis (Stamp, 2003; Macías *et al.*, 2007).

O isolamento da morfina a partir de sementes de papoula em 1806 por Friedrich Wilhelm Sertürner marca o início do estudo de compostos secundários. Em uma das definições mais clássicas, Sachs (1873) define metabólitos secundários como compostos formados durante o metabolismo que não são utilizados na formação de novas células (Sachs, 1873 *apud* Hartmann, 2007).

Antes considerados subprodutos inertes do metabolismo primário, rejeitos metabólicos, atualmente são considerados componentes dinâmicos indispensáveis na estratégia de sobrevivência das plantas em diversos ambientes, compreendendo mais de 200.000 estruturas químicas diferentes (Hartmann, 2007). Os metabólitos secundários auxiliam nos processos de reprodução, dispersão, proteção contra herbívoros e patógenos e

alelopatia, estando diretamente ligados ao sucesso da planta em ocupar o ambiente e sobreviver a estresses. De acordo com as estruturas químicas dos compostos, os metabólitos secundários podem ser classificados convencionalmente em três grandes grupos: terpenos, compostos fenólicos e compostos nitrogenados (Taiz & Zeiger, 2010). Dentro do grupo dos compostos nitrogenados encontra-se o grupo dos alcalóides. Alcalóides ditos verdadeiros são derivados de aminoácidos e são classificados de acordo com o aminoácido precursor de sua origem, sendo os alcalóides indólicos derivados do triptofano.

1.1.1. Alcalóides monoterpeno indólicos

A principal função descrita para o grupo dos compostos nitrogenados, principalmente alcalóides, glicosídeos cianogênicos e aminoácidos não-protéicos, é a proteção contra herbívoros, por conferir sabor amargo, gerar proteínas não-funcionais após ingestão ou, ainda, afetar o sistema nervoso central (Gordon-Weeks e Pickett, 2009). Este grupo inclui compostos que possuem pelo menos um átomo de nitrogênio em sua estrutura, sendo classificados como alcalóides quando este nitrogênio está contido em um anel heterocíclico. Alcalóides geralmente apresentam características alcalinas e, em pHs comumente observados no citosol (pH 7,2) e vacúolo (pH 5-6), o átomo de nitrogênio está protonado, portanto solúvel em água (Taiz *et al.*, 2015).

Alcalóides monoterpeno indólicos são alcalóides de origem sintética mista, sendo a rota padrão de síntese a condensação da triptamina (fração indólica, rota chiquimato/ triptofano) com a secologanina (fração terpênica, rota metileritritol fosfato/ piruvato) pela ação da enzima estrictosidina sintase. Cabe ressaltar, porém, que nem sempre a fração terpênica é oriunda da secologanina, mas de outros monoterpenos; nestes casos, a condensação das duas frações é realizada por uma enzima estrictosidina sintase-*like* (Matsuura e Fett-Neto, 2015).

Alguns exemplos bem conhecidos de alcalóides indólicos são a vincristina e a vinblastina de *Catharanthus roseus*, com ação antitumoral, e a reserpina de *Rauwolfia serpentina*, utilizada no tratamento de problemas neurológicos. Estudos demonstraram a possibilidade do uso de alcalóides indólicos, e derivativos indólicos de origem semissintética, como esqueleto ou arcabouço molecular visando explorar suas propriedades neuroprotetoras;

os anéis indolil-hidantoina e indolilmetil-tio-hidantoina podem ser bons candidatos no desenvolvimento de inibidores da enzima monoaminooxidase A (Klein-Junior *et al.*, 2014).

1.1.2. Ciclotídeos

Por convenção, uma proteína é composta por uma cadeia de aminoácidos conectados por ligações peptídicas contendo pelo menos 40 resíduos de aminoácidos, podendo chegar a mais de 10.000 aminoácidos (Stoker, 2015). Abaixo de 40 resíduos de aminoácidos, as cadeias são classificadas como peptídeos. A convenção para se qualificar como proteína também geralmente se refere a sequências apresentando uma estrutura terciária estável (Lodish, 2004).

Dentre os peptídeos circulares que ocorrem naturalmente nas plantas, os ciclotídeos (17-39 aminoácidos) (www.cybase.org.au) compreendem o maior grupo. Ciclotídeos são caracterizados por possuírem três ligações dissulfeto arranjadas em uma conformação chamada "nó de cisteina" (Craik *et al.*, 1999) (Figura 1) que, somada à natureza circular do peptídeo, proporciona alta estabilidade, sendo resistente à quebra química ou enzimática e à desnaturação por calor (Colgrave & Craik 2004). No reino vegetal, os ciclotídeos foram encontrados em 7 famílias: Rubiaceae, Apocynaceae, Cucurbitaceae, Solanaceae, Violaceae, Fabaceae e Poaceae, e através de análises *in silico* estima-se a presença dos mesmos em mais 6 famílias (Zhang *et al.*, 2015). Em diferentes graus, alguns ciclotídeos foram caracterizados (Gruber *et al.*, 2008; Gerlach *et al.*, 2013).

Alguns ciclotídeos são expressos constitutivamente enquanto outros tem expressão variada ao longo do ano, mas pouco se sabe sobre a regulação da expressão gênica de ciclotídeos até o momento (Shafee *et al.*, 2015). Sua síntese envolve clivagem, ciclização e formação de ligações dissulfeto de domínios de ciclotídeos contidos em proteínas precursoras; esse processamento ocorre no retículo endoplasmático e no vacúolo. Para tanto, o precursor de um ciclotídeo contém basicamente três partes: 1) uma porção N-terminal contendo sequências de direcionamento ao vacúolo; 2) o domínio ciclotídeo, contendo o motivo "nó de cisteína"; 3) uma porção C-terminal, de estrutura desconhecida, necessária para o processo de ciclização; além disso um peptídeo sinal hidrofóbico direciona o precursor ao retículo endoplasmático (Shafee *et al.*, 2015).

Atividades descritas para esses compostos incluem ação uterotônica, anti-HIV (vírus da imunodeficiência humana), antimicrobiana, inseticida e moluscicida (Gran *et al.*, 2000; Gustafson *et al.*, 2004; Tam *et al.*, 1999; Barbeta *et al.*, 2008; Plan *et al.*, 2008). Além disso,

devido a características de estabilidade, podem ser usadas como esqueleto ou arcabouço molecular no projeto de drogas altamente estáveis (Thorstholm & Craik 2011). As evidências apontam que os ciclotídeos têm uma função de defesa principalmente contra herbívoros e o principal mecanismo de ação proposto envolveria a formação de poros em membranas no intestino dos animais (Huang *et al.*, 2009). Foi sugerido que os ciclotídeos ultrapassam as proteínas da classe das defensinas em número e diversidade (Trabi *et al.*, 2004).

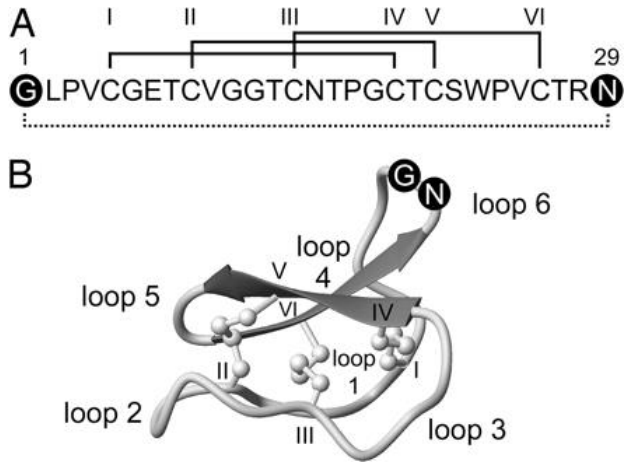

Figura 1. Sequência de aminoácidos (A) e estrutura (B) do primeiro ciclotídeo isolado e descrito, kalata B1, de *Oldenlandia affinis* (Rubiaceae). Fonte: Barbeta *et al.*, 2008**.**

1.2 *Psychotria*

A família Rubiaceae abriga o gênero *Psychotria* (Figura 2), sendo este o maior grupo dentro desta família e um dos mais ricos em números de espécies dentro das angiospermas. Mais de 1800 espécies distribuídas mundialmente integram o gênero (Nepokroeff *et al.*, 1999; Nepokroeff *et al.*, 2003; Davis *et al.*, 2009).

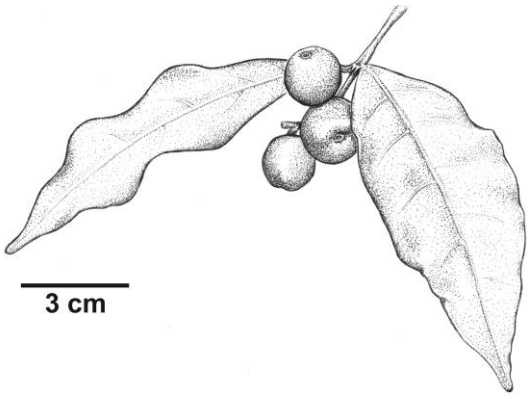

Figura 2. Algumas características compartilhadas pelas espécies do gênero *Psychotria*: folhas opostas, estípulas interpeciolares, ovário ínfero e florescências determinadas. Fonte: Matsuura *et al.*, 2013. Ilustração: Flávio Augusto Pretto.

Frequentemente, as espécies de *Psychotria* habitam regiões de sub-bosque e apresentam um porte arbustivo. Várias delas acumulam alcalóides indólicos, sendo estes uma ferramenta importante no auxílio à taxonomia deste grupo, uma vez que diferenças morfológicas são, por vezes, sutis entre as espécies (Nepokroeff *et al.*, 1999). As espécies neotropicais são caracterizadas pela presença de alcalóides monoterpeno indólicos e pertencem ao subgênero Heteropsychotria. *P. ipecacuanha*, apesar de sua distribuição, não pertence ao clado Heteropsychotria e compartilha a presença de alcalóides iridóides derivados de dopamina e não de triptamina, com *P. borucana* (Nepokroeff *et al.*, 1999) e *P. klugii* (Muhammad *et al.*, 2003).

As moléculas bioativas sintetizadas por *Psychotria* spp. são diversas e incluem peptídeos, alcalóides, pigmentos, naftoquininas e benzoquinonas (Beretz *et al.*, 1985; Hayashi *et al.*, 1987; Witherup *et al.*, 1994; Glinski *et al.*, 1995; Solis *et al.*, 1995). Tais metabólitos apresentam várias atividades biológicas, entre elas: antibiótica, antifúngica, antiviral, antiinflamatória, citotóxica e antiparasitária (Rasolonjanahary *et al.*, 1995; Dunstan *et al.*, 1997; Khan *et al.*, 2001; Kuo *et al.*, 2001; Muhammad *et al.*, 2003; Faria *et al.*, 2010).

Atualmente *P. ipecacuanha* é denominada *Carapichea ipecacuanha*. *C. ipecacuanha* (atual *Carapichea ipecacuanha*) está entre as espécies mais estudadas de *Psychotria* devido aos alcalóides isoquinolínicos emetina e cefalina por ela sintetizados, ambos com propriedades eméticas (Sousa *et al.*, 1991). A emetina tem também comprovado seu efeito no tratamento de amebíase (Lewis & Elvin-Lewis, 1977), porém apresentando alguns efeitos colaterais indesejados como fraqueza muscular e danos cardíacos. Recentemente, foi descoberto que este alcalóide é capaz de penetrar em partículas virais intactas, ligar-se à

transcritase reversa e inibir a replicação do vírus HIV, sendo um forte candidato como fármaco anti-HIV (Valadão *et al.*, 2015).

No Sul do Brasil, o estudo fitoquímico do gênero *Psychotria* iniciou com a avaliação de seis espécies (*Psychotria brachyceras*, *P. carthagenensis*, *P. leiocarpa*, *P. myriantha*, *P. suterella* e *P. umbellata*), algumas das quais revelaram atividade analgésica (Leal, 1994). Estes achados estimularam a busca por novas atividades farmacológicas, a identificação dos compostos responsáveis pelas mesmas e a melhor compreensão de seus processos de síntese.

P. brachyceras, *P. leiocarpa*, *P. umbellata* e *P. carthagenensis* são encontradas no sub-bosque de florestas tropicais e subtropicais do Brasil, Argentina e Paraguai (Smith & Downs, 1956; Dillenburg & Porto, 1985), muitas vezes compartilhando o mesmo habitat. Observações a campo das quatro espécies indicaram uma elevada taxa de predação em *P. carthagenensis* (Figura 3) em comparação às demais, principalmente *P. leiocarpa* e *P. brachyceras*. Análise dos principais compostos de defesa conhecidos revelaram a presença de alcalóides como os mais prováveis fatores envolvidos, sendo *P. carthagenensis* a única espécie a não apresentar alcalóides (Leal & Elisabetsky, 1996). Um estudo preliminar identificou a presença de ciclotídeos em *P. brachyceras*, enquanto *P. carthagenensis* não mostrou indícios de possuir estes peptídeos (Gruber *et al.*, 2008). As ausências simultâneas de alcalóides e ciclotídeos nesta última espécie poderiam explicar a maior predação observada.

Figura 3. Evidências de predação em *Psychotria carthagenensis*. Fotografia: Hélio Nitta Matsuura.

Os alcalóides estão presentes em teores elevados nas partes aéreas destas plantas (Kerber *et al.*, 2001; Henriques *et al.*, 2004; Paranhos *et al.*, 2005). Enquanto os alcalóides

psicolatina (*P. umbellata*) e N,*β*-D-glicopiranosil vincosamida (GPV) (*P. leiocarpa*) apresentam uma maior concentração (atingindo 4% e 2,5% do peso seco foliar, respectivamente) e um padrão de acúmulto tipo fitoanticipina, o alcalóide braquicerina (*P. brachyceras*) apresenta um perfil induzível, mais próximo ao de uma fitoalexina, com um teor basal de 0,2% do peso seco, que pode aumentar até 10 vezes frente a estresses. Apesar dos altos teores, os alcalóides indólicos de *Psychotria* parecem não estar envolvidos diretamente em sua proteção contra herbívoros (Matsuura *et al.*, 2013; Porto *et al.*, 2014), mas atuar fundamentalmente como antioxidantes, detoxificando o acúmulo de espécies reativas de oxigênio (as estruturas destes alcalóides podem ser encontradas no material suplementar do Capítulo 2 desta Tese). Este perfil explicaria mudanças na expressão gênica relacionada ao metabolismo alcaloídico (Nascimento *et al.*, 2013a) e no acúmulo de braquicerina frente a variadas fontes de estresse, incluindo dano mecânico e jasmonato (Gregianini *et al.*, 2004), radiação ultravioleta B (UV-B) (Gregianini *et al.*, 2003), estresse osmótico, presença de metais pesados e presença de ácido absícico (Nascimento *et al.*, 2013b).

Os ciclotídeos são, provavelmente, os principais compostos envolvidos na proteção contra herbívoros nessas espécies de *Psychotria*, uma especulação apoiada no screening por ciclotídeos na família Rubiaceae que revelou a presença desses peptídeos em *P. brachyceras*, mas não na altamente predada *P. carthagenensis* (Gruber *et al.*, 2008). Claramente, mais investigações são necessárias para fundamentar ou refutar esta possibilidade, sendo um dos objetivos do presente trabalho.

Justificativa

Dentre os alcalóides monoterpeno indólicos de *Psychotria* do Sul do Brasil, GPV apresenta importantes lacunas no entendimento da regulação espaço-temporal de seu acúmulo em *P. leiocarpa*. Embora tenha sido verificado que sua produção é dependente de luz em plântulas cultivadas *in vitro*, não era sabido como plântulas respondem às transições de estados fotomorfogênicos e estiolados, nem tampouco qual o efeito da qualidade de irradiância na dinâmica do alcalóide. Do mesmo modo a distribuição órgão-específica de GPV em estruturas vegetativas e reprodutivas não era conhecida. Por outro lado, embora GPV tenha sido caracterizado como antioxidante, não tinha sido examinada a eficácia do mesmo em comparação direta com demais alcalóides de *Psychotria* estruturalmente semelhantes e controles positivos. A capacidade de GPV em realizar o quenching de espécies reativas de oxigênio (ROS) *in vivo* e seus efeitos indiretos também não tinha sido examinada.

Recentemente, os ciclotídeos mostraram possuir propriedades farmacológicas de grande interesse, incluindo ação anti-HIV, antitumoral e antibacteriana e, devido à sua estrutura, mostraram-se ferramentas interessantes na projeção de drogas estáveis. A pesquisa na área de projeção de drogas cresce rapidamente; porém, até o momento, pouco se sabe sobre os mecanismos regulatórios da síntese destes peptídeos. O presente trabalho teve, dentre seus objetivos, identificar peptídeos e elucidar a regulação da síntese de ciclotídeos em algumas espécies de *Psychotria* do Sul do Brasil e sua cinética de acúmulo frente a diversos estresses. Estudos dessa natureza não haviam sido realizados. Adicionalmente, a presença de ciclotídeos em *P. brachyceras* coincide com uma menor taxa de predação quando comparada a *P. carthagenensis*, uma espécie que não apresenta ciclotídeos, sugerindo uma possível participação destes compostos na proteção da planta.

Objetivos

Em vista do exposto, neste trabalho buscou-se verificar e melhor compreender a dinâmica de acúmulo espaço-temporal de GPV em *P. leiocarpa* e quantificar de modo comparativo suas propriedades antioxidantes. Também se almejou avaliar a presença de ciclotídeos em algumas espécies de *Psychotria* do Sul do Brasil, além de atribuir uma provável função *in planta* para estes peptídeos. Foram objetivos específicos do trabalho:

- Analisar a dinâmica do alcalóide GPV de *P. leiocarpa* em relação a mudanças de quantidade e qualidade de irradiância e distribuição órgão-específica em estruturas vegetativas e reprodutivas.

- Realizar a detecção e, quando presentes, caracterizar os ciclotídeos de *P. brachyceras, P. leiocarpa* e *P. umbellata*;

- Avaliar a regulação da síntese dos ciclotídeos de *P. brachyceras* e *P. leiocarpa* frente a estresses simulados relacionados à herbivoria e presença de patógenos;

- Sugerir a possível função ecológica dos ciclotídeos dessas espécies de *Psychotria*.

Organização dos capítulos

No **Capitulo I** são revisadas as principais funções e mecanismos de ação de alcalóides tóxicos de origem vegetal. Classicamente, os alcalóides apresentam uma função de defesa contra herbívoros; porém, tem sido verificado que, apesar de vários indícios acerca dos alcalóides majoritários de três espécies de *Psychotria* apontarem para uma função anti-herbivoria, os mesmos não cumprem essa função (*Anexos III e IV*), sendo seu alto teor observado possivelmente relacionado à suas propriedades antioxidantes.

No **Capítulo II** é descrita a dinâmica de acúmulo espaço-temporal de GPV em *P. leiocarpa*, regulado pela presença e qualidade de luz e com distribuição órgão-específica, tanto reprodutiva como vegetativa. As propriedades antioxidantes do alcalóide são também examinadas, sugerindo que podem estar envolvidas na proteção de estruturas e/ou fases de desenvolvimento mais sensíveis da planta.

No **Capítulo III** é examinado como a elicitação de alcalóides indólicos pode estar associada ao estresse oxidativo gerado durante a resposta contra estresses gerais, bióticos e abióticos, e possíveis mecanismos envolvidos, reforçando a possibilidade de muitos destes alcalóides atuarem parcial ou predominantemente como antioxidantes, além de possíveis aplicações biotecnológicas destas constatações para produção em escala de alcalóides bioativos desta classe.

No **Capítulo IV**, a presença de ciclotídeos em espécies de *Psychotria* é explorada, constatando-se abundância destes peptídeos em algumas espécies e ausência em outras. Este fato oferece uma possível resposta para o questionamento inicial das diferenças observadas entre as preferências de predadores a algumas espécies. De fato, um ciclotídeo inédito na literatura apresentou eficiente atividade inseticida.

Capítulo I: Capítulo de livro publicado.

Plant alkaloids: main features, toxicity, and mechanisms of action

Livro: Plant Toxins
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Plant Alkaloids: Main Features, Toxicity, and Mechanisms of Action

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Abstract

Alkaloids are one of the largest groups of plant secondary metabolites, being present in several economically relevant plant families. Alkaloids encompass neuroactive molecules, such as caffeine and nicotine, as well as life-saving medicines including emetine used to fight oral intoxication and the antitumorals vincristine and vinblastine. Alkaloids can act as defense compounds in plants, being efficient against pathogens and predators due to their toxicity. Fast perception of aggressors and unfavorable environmental conditions, followed by efficient and specific signal transduction for triggering alkaloid accumulation, are key steps in successful plant protection. Toxic effects, in general, depend on specific dosage, exposure time, and individual characteristics, such as sensitivity, site of action, and developmental stage. At times, toxicity effects can be both harmful and beneficial depending on the ecological or pharmacological context. Different strategies are used to study alkaloid metabolism and accumulation. An efficient approach is to monitor gene expression, enzyme activities, and concentration of precursors and of the alkaloid itself during controlled attacks of pathogens and herbivores or upon the simulation of their presence through physical or chemical stimulation. Detailed understanding of alkaloid biosynthesis and mechanisms of action is essential to improve production of alkaloids of interest, to discover new bioactive molecules, and to sustainably exploit them against targets of interest, such as herbivores, pathogens, cancer cells, or unwanted physiological conditions.

Keywords

Alkaloid; Antioxidant; Antitumoral; Herbivory; Pathogen

Introduction

Natural products have been exploited by humans for thousands of years, used as foods, drugs, antioxidants, flavors, fragrances, dyes, insecticides, and pheromones, improving our health, enhancing crop production, unraveling complex ecological interactions, and shaping our way of life. Alkaloids are among the largest groups of secondary metabolites, being extremely diverse in terms of structure and biosynthetic pathways, including more than 20,000 different molecules distributed throughout approximately 20 % of known vascular plants (Yang and Stöckigt 2010).

Alkaloids are low-molecular-weight nitrogen-containing compounds and, due to the presence of a heterocyclic ring containing a nitrogen atom, are typically alkaline. Alkaloids are known by their numerous pharmacological effects on vertebrates. These metabolites can be divided into different classes according to their precursor (e.g., indole alkaloids are alkaloids derived from tryptophan), encompassing

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more than 20 different classes (e.g., pyrrolidine alkaloids, tropane alkaloids, piperidine alkaloids, pyridine alkaloids, quinolizidine alkaloids, and indole alkaloids, among others) (Yang and Stöckigt 2010).

The presence of alkaloids and other secondary metabolites in plants enhances plant reproductive rates, either by improving defenses against biotic and abiotic stresses or by affecting pollinators and seed/fruit disperser visitation. Defensive strategies include predator repellence by toxicity or bitterness taste or damage repair by antioxidant system (Vilariño and Ravetta 2008; Matsuura and Fett-Neto 2013). Flower visitors can be attracted by stimulant properties of some alkaloids, whereas visit duration can be controlled by nonlethal toxicity (Irwin et al. 2014). This and several other examples of metabolic versatility lead to significant improvement in survival rates for plants and, at the same time, provide important pharmacological activities for the human therapeutic arsenal, such as antioxidant compounds, antitumoral drugs, analgesics, anti-inflammatories, and stimulants (Yang and Stöckigt 2010).

The major role described for plant alkaloids in the scientific literature revolves around protection against herbivores, for several alkaloids present characteristics such as bitter flavor, disruption of protein function after ingestion and metabolization, and central nervous system alteration (Harborne 1993). To minimize self-intoxication risk, defense compounds are often stored in the vacuole or apoplastic compartment, showing limited metabolic activity (Mithöfer and Boland 2012).

Toxic Alkaloids

Alkaloids are among the most important drugs in human history. The isolation of the alkaloid morphine by Friedrich Wilhelm Sertürner in 1806 is regarded as the "formal" start of plant secondary metabolism (Hartmann 2007). It is widely accepted that the main role of alkaloids in plants is toxicity against predators and pathogens. The same toxic properties observed in the plant defense scenario can often be used in prospection for new drugs. For example, a very specific toxicity may be used to fight certain tumor cell types, or also be used to control specific microorganisms or pests (Yang and Stöckigt 2010; Lee et al. 2014).

Different uses of plant alkaloids have been reported during history, including medicinal, therapeutic, recreational, and religious. The use of plant alkaloids from distinct classes to alter senses has been known since ancient times due to the ability of several of these molecules to modulate the human central nervous system (CNS). The use of opium poppy (*Papaver somniferum*) latex has been recorded as early as 1400 to 1200 B.C. in the Eastern Mediterranean. The roots of *Rauvolfia serpentina* have been used in India since approximately 1000 B.C. The Greek philosopher Socrates was executed in 399 B.C. by drinking an extract of hemlock (*Conium maculatum*). The Egyptian queen Cleopatra used extracts of henbane (*Hyoscyamus*), which contain atropine, to dilate pupils and appear more seductive. Tropane alkaloids from several Solanaceae species were used in sorcery by "witches" during the Middle Ages (Croteau et al. 2000; Evans and Hofmann 2006).

Presently used toxic or potentially toxic alkaloids include caffeine, constituent of daily foods and beverages containing coffee (*Coffea arabica*), tea (mostly *Camellia sinensis*), or cocoa (*Theobroma cacao*), consumed for mental alertness, as well as physical training enhancement; nicotine in cigars, cigarettes, and pipes (*Nicotiana tabacum*), a CNS stimulant; morphine (*Papaver somniferum*), one of the most powerful known analgesics; and codeine found in the same species, a sedative and cough suppressant. Illicit psychoactive drugs that cause massive social and economic problems, such as cocaine (*Erythroxylum* sp.) and its derivatives (Koleva et al. 2012; Senchina et al. 2014), are also contemporary toxic alkaloids. Strychnine, from *Strychnos nux-vomica*, is a very powerful tetanic poison, acting as competitive antagonist at glycine receptors. Its main current uses are as rat poison and in homeopathy (Croteau et al. 2000).

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For crop management purposes, the presence of alkaloids of low toxicity to humans can be an advantage by keeping herbivores away. For example, *Lupinus* species with higher quinolizidine content, thus less palatable, require less pesticide application (Vilariño and Ravetta 2008). Consistently, production of tomatoes with very low contents or lacking solanine, selected for appropriate human consumption, requires larger amounts of pesticides. Crops that did not undergo long-term artificial selection, often focused essentially on edible organs for human food supply, can still bear useful defensive traits, thereby requiring less agricultural inputs to keep herbivores and competitors away. There is also evidence for allelopathic activity of some plant alkaloids against target species mostly in laboratory assays. Inhibition of *Lactuca sativa* and *Lepidium sativum* seedling growth by berberine, sanguinarine, and gramine, among other alkaloids, has been recorded. Although less phytotoxic than essential oil terpenes, for instance, quinine, cinchonidine, nicotine, boldine, lobeline, coniine, and harmaline proved phytotoxic to *Lemna gibba*, causing death or chlorosis (Wink and Twardowski 1992). Whether alkaloid phytotoxicity could be used in weed control remains to be tested.

Some animals can stock toxic alkaloids indirectly acquired from plants, as is the case of poison frogs (Dendrobatidae) from South and Central America forests. The source of alkaloids is alkaloid-containing arthropods that previously accumulated toxins presumably by feeding on toxic alkaloid-containing plants. The presence of plant alkaloids chimonanthine, calycanthine, and nicotine, or its enantiomers, has been reported in the skin of Dendrobatidae frogs (Saporito et al. 2012). Native Indians from the Amazon use the secretion of poison frogs to contaminate the point of darts used in hunting and rapidly kill or impair birds and little mammals. Bufonidae frogs were believed to produce alkaloids instead of accumulating them from a food source, but recent studies showed that Bufonidae frogs also obtain alkaloids from the diet (Hantak et al. 2013). Some species of *Phyllobates* (Dendrobatidae) can secrete batrachotoxins, which are the most potent known non-peptide neurotoxins (Zhang et al. 2014). Pyrrolizidine alkaloids of species of *Crotalaria* (rattlebox), which serve as hosts to the moth *Utetheisa ornatrix* (bella moth), can be stored by larvae, making them poisonous and frequently repellent to predators, a feature that remains through the pupae and adult stages. In addition, the alkaloids and biotransformation products of these are given to females as a nuptial gift, which is transferred to eggs, presumably making these protected against predators (Eisner 2003).

Toxicity to Humans and Other Vertebrates

Animal intoxication by alkaloids is mostly caused by accidental ingestion of food contaminated with alkaloid-containing plants. Clearly, the amount of ingested alkaloid and the sensitivity of the target animal are key factors leading to intoxication. Some alkaloids can be extremely harmful to mammals, which is the case of the steroidal alkaloid cyclopamine in lambs, identified as the compound in *Veratrum californicum* (Liliaceae) responsible for teratogen effects resulting in craniofacial birth defects causing a cyclops aspect in offspring of sheep grazing *V. californicum* (Fig. 1). First reports on this phenomenon occurred during the late 1960s in the western United States (Lee et al. 2014).

Plants containing tropane alkaloids (TAs) are found in numerous and important plant families such as Solanaceae, Brassicaceae, Erythroxylaceae, Convolvulaceae, and Euphorbiaceae. TAs are alkaloids derived from ornithine, and in many parts of the world, TA-containing plants have been used for folkloric and medicinal purposes due to their powerful anticholinergic (e.g., scopolamine) and hallucinogenic effects (e.g., hyoscyamine and atropine), causing constipation, photophobia, pupil dilatation, vision disturbance, and dryness of upper digestive and respiratory tract mucosa. Contaminations with TAs often occur via ingestion of food containing *Datura*, which accumulates high concentrations of scopolamine and hyoscyamine (Koleva et al. 2012).

In *Solanum* plants (Solanaceae), the commonly present glycoalkaloids, solanine and chaconine, can be found in species such as nightshades (*S. nigrum*), potato (*S. tuberosum*), tomato (*S. lycopersicum*),

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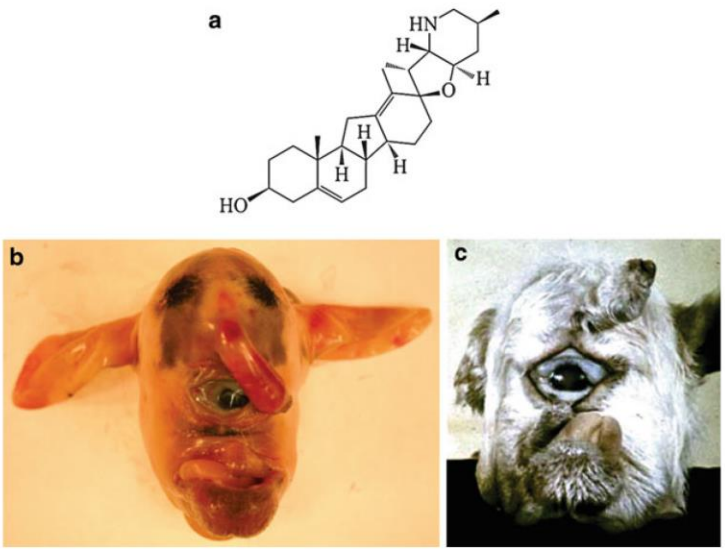

Fig. 1 (**a**) Structure of the toxic alkaloid cyclopamine from *Veratrum californicum*; (**b**, **c**) lambs with cyclops phenotype due to alkaloid ingestion by their mother (Adapted with permission from Lee et al. (2014). Copyright (2014) American Chemical Society)

eggplant (*S. melongena*), pepper (*Capsicum annuum*), and petunia (*Petunia* sp.), carrying fungicidal and pesticidal properties participating in plant defense mechanisms. Poisoning by solanine ingestion primarily causes gastrointestinal and neurological disorders. The mechanism of action can be due to inhibition of acetyl cholinesterase and calcium transport, which occur in micromolar range. A synergic effect that increases toxicity is likely to be observed when solanine and chaconine are combined (Yamashoji and Matsuda 2013).

The plant families Asteraceae, Boraginaceae, and Fabaceae often produce pyrrolizidine alkaloids (PAs), which are also ornithine-derived alkaloids, estimated to be present in more than 6,000 plants and known to be efficient against predators, including human and livestock (Shimshoni et al. 2015). PAs' acute and chronic liver toxicity in humans and other animals is well known, and some symptoms of acute PA poisoning are abdominal pain, nausea, vomiting, diarrhea, and edema (Koleva et al. 2012). Highly toxic carcinogenic and genotoxic effects are reported as the main mechanism of action of PAs (Shimshoni et al. 2015). Food contaminated with PAs, mostly esters of 1-hydroxymethyl-1,2-dehydropyrrolizidine, include vegetables, grain-derived products, eggs, honey, offal, and milk, due to contamination of the grains by seeds and/or plant fragments from PA-containing weeds growing in the crops used for animal feeding or human consumption (Koleva et al. 2012). Grazing animals will generally avoid PA-containing plants; however, in unfavorable conditions, such as overgrazed pastures and favored toxic weed development caused by drought, a behavior of PA-containing weed consumption can be observed (Shimshoni et al. 2015). A screening of 350 plant-derived PAs showed that approximately half of them were hepatotoxic and several were carcinogenic (Cushnie et al. 2014). In addition to PAs, iridoid glycoside (IG) presence also confers plant resistance, and a combined defense is often common and most effective for plants to increase protection (Shimshoni et al. 2015).

Some quinolizidine alkaloids, as the case of lupin alkaloids, are toxic to humans in acute doses, which may occur when consuming lupin beans that were not previously debittered, causing dry mouth, blurry vision, facial flushing, and confusion (Koleva et al. 2012).

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To adult livestock animals, piperidine alkaloids (derived from lysine) can be acutely toxic causing musculoskeletal deformities in neonatal individuals. Signs of acute intoxication by piperidine alkaloids in livestock include frequent urination and defecation, muscle weakness, tachycardia, ataxia, muscle fasciculations, collapse, and death by respiratory failure. The teratogenic effect of some piperidine alkaloids, such as ammodendrine, N-acetylhystrine, anabaseine, coniine, and γ-coniceine, include multiple congenital contracture deformities and cleft palate in pigs, goats, cattle, and sheep. Poisonous plants containing teratogenic piperidine alkaloids include some *Lupinus* sp., *Laburnum* sp., *N. tabacum*, *N. glauca*, and *Conium maculatum* (Green et al. 2012).

Taxines are a mixture of active alkaloids from yew trees (*Taxus* sp., Taxaceae), which have been implicated in several animal and human poisonings with predominant cardiovascular effects. Although some taxines are related to the antitumor drug Taxol, they are distinct molecules. Toxicity of the yew genus has been known since the second century B.C., particularly among Celts and related cultures (Wilson et al. 2001).

Excess of daily-consumed metabolites such as caffeine can also be considerably toxic. Some overdose symptoms include tachycardia, arrhythmia, convulsions, vomiting, and eventually coma and death. The average caffeine content in a cup of coffee or tea is between 40 and 150 mg, and medicinal/fitness supplements may contain some 100–400 mg. Lethal caffeine overdoses are typically in excess over 5 g in adults and are relatively rare, generally occurring by accidental causes (Kerrigan and Lindsey 2005).

Due to stimulatory and addictive effects of nicotine from tobacco, the popularity of tobacco products and their widespread use remain, causing billions of people around the world to use it, despite the fact that almost all users are aware of the numerous negative health and economic impacts of smoking (Dewey and Xie 2014). Nicotine is also important as a treatment to help quit smoking, in the form of skin patches and gums.

Cocaine and its derivatives are extremely addictive and harmful drugs, with devastating effects in health and behavior of users, carrying economical and social disorders to society. Chewing coca leaves has been a centuries-old practice of Andean native people. The presumed effects of this practice are related to improved physical performance; in fact, this information has found some support in controlled experiments involving physical exercises. However, the beneficial effects may not be related to the minute amounts of cocaine ingested by leaf chewing, but rather to flavonoids or other constituents that could function as adaptogens (Casikar et al. 2010).

Adaptations of some animals to tolerate plant alkaloids, and even store these compounds, such as alkaloid-accumulating poison frogs, require specialized strategies including storage of the defensive compound in specialized structures (dermal granular glands, located at the dorsum), conversion of the metabolite into a less toxic form prior to storage (e.g., conversion of pyrrolizidine alkaloids to N-oxides), and changes at molecular level in ion channel sites or receptors to avoid self-intoxication (Saporito et al. 2012).

Anti-herbivory and Pollinator Interactions (Focus on Insects)

Plant arsenals to cope with herbivores include repellent, antinutritive, and toxic compounds. Some examples are alkaloids, cyanogenic glycosides, glucosinolates, terpenoids, and also macromolecules such as proteinase inhibitors and cyclotides, solid inclusions (raphides and druses), resins, and latex.

Alkaloid-mimicking sugars are efficient inhibitors of several sugars and glycosidases metabolizing enzymes by inhibition of trehalase in some tissues and sucrose in the midgut, leading to toxic effects and affecting growth once the insect becomes disabled to use threalose or uptake sucrose. Colchicine from *Colchicum autumnale* (Colchicaceae) is toxic to honey bee (*Apis mellifera*) and inhibits microtubule polymerization by binding to tubulin and inhibiting mitosis (Mithöfer and Boland 2012). Pollinators are exposed to a diverse array of alkaloids, similar to grazing animals, since secondary metabolites can also be

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present in plant reproductive tissues, as well as in nectar and pollen. Some negative consequences, such as reduced ovary development, mobility, and survivorship, are documented for several pollinators visiting alkaloid-containing plants, but, in some cases, secondary compounds present in nectar can be beneficial to the pollinator, reducing gut pathogens. In fact, low concentrations of some alkaloids can attract pollinators (Irwin et al. 2014). A strategy of accumulating both attractant (e.g., sugars and volatile phenolics) and repellent (e.g., alkaloids) compounds in the nectar observed in *N. attenuata* results in benefits to the plant by decreasing pollinator visitation time and increasing the number of visited flowers (Brandenburg et al. 2009). The presence of low concentrations of caffeine in nectar (below its bitterness threshold) of some Rubiaceae and Rutaceae has been shown to potentiate the pollinator memory of reward by acting as an adenosine receptor antagonist, stimulating more visits to the same flower (Wright et al. 2013).

Plant alkaloid toxicity can be quite diversified, but often involves neurotoxicity or cell signaling disruption (Mithöfer and Boland 2012). Sanguinarine from *Sanguinaria canadensis* (Papaveraceae) presents multiple toxic effects. This alkaloid inhibits choline acetyltransferase, affecting neurotransmission; it also affects several other neuroreceptors and DNA synthesis. Caffeine found in *C. arabica* (Rubiaceae) and various other plant species is often toxic and paralyzes insects feeding on the plant. Caffeine inhibits phosphodiesterase activity and promotes increase in intracellular cyclic AMP level. In vertebrates, the interaction of the alkaloid with adenosine receptors of the nervous system is responsible for stimulating effects. Nicotine effect lies on the ability of some alkaloids to bind various neuroreceptors and block or displace endogenous neurotransmitters. Nicotine acts as an agonist or antagonist targeting nicotinic acetylcholine receptors in insects, which are the most abundant excitatory postsynaptic receptors, causing continual neuronal excitation, leading to insect paralysis and death (Dewey and Xie 2014). Nicotine accumulation is triggered by herbivore attack, which leads to increased jasmonic acid (JA) levels in wounded leaves, signaling for nicotine synthesis in roots, and subsequent transport of the alkaloid to aerial parts (Mithöfer and Boland 2012).

"Friendly" Toxicity

The alkaloid mechanism of action is complex, meaning that toxicity observed in insects, for example, is not necessarily the same to other animals. Key aspects related to toxicity symptoms include the amount of active metabolite, the organ that it is in contact with, and particular characteristics of the target organism. Understanding alkaloid metabolism and action can lead to useful molecules for human health and crop production.

Some important drugs of the therapeutic arsenal that are plant alkaloids include morphine to treat severe pain; emetine and cephaeline as antidotes for intoxication; caffeine with its stimulant properties; quinine used due to its antimalarial properties and bitter taste; the antitumorals vincristine, vinblastine, and camptothecin; anti-arrhythmic ajmaline; antihypertensives serpentine and ajmalicine; antimicrobials berberine and sanguinarine; antitussive noscapine; vasodilator papaverine; and the muscle relaxant tubocurarine (Yang and Stöckigt 2010).

Alkaloids are also consumed to improve immune functions, nutrition, and physical performance, being present in daily foods, beverages, and supplements. Some examples include the caffeine from coffee (or guaranine and mateine from other plants) with antioxidant, anti-inflammatory, and stimulatory properties; theobromine and paraxanthine from cocoa as antioxidants; and gingerol and shogaols (phenolic alkanones) present in ginger bearing antioxidant, anti-inflammatory, antimicrobial, and antitumoral properties (Senchina et al. 2014; Han et al. 2015). Mitochondria are the major intracellular sources of reactive oxygen species (ROS) in animal cells. Conjugates of the plant alkaloids berberine and palmatine with the antioxidant plastoquinone can be used as a strategy in therapies focusing mitochondria-targeted antioxidant activity (Apostolova and Victor 2015). Various alkaloids display antioxidant properties, some of which being effective skin sunscreens (Machowinski et al. 2006; Ahsan

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et al. 2007). Some alkaloids may have a major role in plants as antioxidants rather than as toxins for herbivores, thereby helping the detoxification of reactive oxygen species generated by different stresses (Matsuura et al. 2014; Porto et al. 2014).

Antibacterial activity is reported for various alkaloid classes, including aaptamine, indole, indolizidine, isoquinoline, piperazine, quinoline, quinolone, agelasine, polyamine, aaptamine-indole, bisindole, indole-quinoline, pyridoacridine, bispyrrole, and pyrrole-imidazole alkaloids (Cushnie et al. 2014). In addition, natural xenobiotics, such as gramine, can prevent cyanobacterial and algal growth, being useful tools in freshwater quality management and ecology (Laue et al. 2014).

Alkaloids previously known as exclusively harmful have often found new uses. Protective and therapeutic effects of solanine treatment were observed in animal breast cancer models, with reduction in tumor size and weight, apoptosis induction, as well as an inhibition of angiogenesis and cell proliferation (Mohsenikia et al. 2013). Cyclopamine has displayed potential as antitumor agent. Cyclopamine teratogenic properties lie on inhibition of the sonic hedgehog (Shh) signaling pathway, which plays a critical role in development of embryos; interestingly, the very same inhibition of Shh signaling is a promising treatment method for several cancer types. Human patients carrying basal cell carcinomas treated with a topical cream containing cyclopamine showed tumor regression and no adverse effects (Lee et al. 2014).

Mechanisms of Action

Alkaloids affect different metabolic systems in animals, and the toxic mechanism of action of alkaloids may vary considerably. Toxicity may arise by enzymatic alterations affecting physiological processes, inhibition of DNA synthesis and repair mechanisms by intercalating with nucleic acids, or affecting the nervous system. Several alkaloids may affect multiple functions (Mithöfer and Boland 2012).

Taxines are calcium channel antagonists, increasing cytoplasmic calcium (Wilson et al. 2001). Pyrrolizidine alkaloid toxic effects are mainly due to their biotransformation into strong reactive pyrrole structures by oxidases from the mammalian liver. The reactive pyrroles act by alkylating nucleic acids and proteins (Cushnie et al. 2014). Alkaloid mechanisms of action as antibacterial agents differ among alkaloid classes. Synthetic quinolone alkaloids may have respiratory inhibition effects; isoquinolines, such as berberine, sanguinarine, protoberberine, and benzophenanthridine, inhibit cell division by perturbing the Z-ring; the phenanthridine isoquinoline alkaloid ungeremine acts by inhibiting nucleic acid synthesis; pergularinine and tylophorinidine, which are indolizidine alkaloids, inhibit nucleic acid synthesis as well, by targeting dihydrofolate reductase (Cushnie et al. 2014).

Plant Alkaloid Accumulation Strategies and Dynamics

Accumulation of defense compounds in plants, originating either from primary (e.g., toxic peptides) or secondary (e.g., alkaloids) metabolism, is closely related to the survival strategy of the organism in the environment by ensuring adequate maintenance of basic primary metabolism activity. In stressful environments, such as those with extreme temperatures, floods, and/or droughts, mechanisms to tolerate freezing and dormancy periods, to prevent loss of water or to deal with anoxia, may also require modifications/specializations in metabolism, besides morphological and anatomical adaptations.

Several biotic and abiotic stressing conditions modulate the induction of alkaloids as well as other secondary metabolites. The presence of herbivores and pathogens; wounding; hormones mimicking herbivore/pathogen attacks, such as JA and salicylic acid (SA); changes in irradiance intensities and

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qualities [e.g., high red/far-red ratio and ultraviolet-B radiation (UV-B)]; temperature; drought; and soil nutrient composition can affect alkaloid concentrations in plants. As for most secondary metabolites, alkaloid accumulation is also often responsive to developmental signals, such as changes associated with flowering and fruit setting (Nascimento and Fett-Neto 2010), as well as with leaf growth (Roepke et al. 2010). The elucidation of alkaloid biosynthetic pathways and the influence of external and developmental signals on them may not only help in understanding the ecological roles of these compounds but also assist in defining strategies to improve their production for pharmacological or agrochemical purposes.

Catharanthus roseus together with *Rauwolfia serpentina* (Apocynaceae) produce a range of important alkaloids such as vincristine, vinblastine, reserpine, ajmaline, ajmalicine, and serpentine and are model plants in MIA (monoterpene indole alkaloid) biosynthesis. Relatively detailed physiological and ecological aspects of MIA production are known for *C. roseus*.

C. roseus has specialized alkaloid accumulation strategies, with an elaborate compartmentalization system involving at least four cell types and further subcellular distribution in various organelles. As expected, such morpho-metabolic organization is seamed together by tight regulated mechanisms of intracellular and extracellular translocation events. This complex spatial organization regulates metabolic fluxes and allows efficient plant defense. *C. roseus* leaf protection is likely ensured by accumulation of toxic MIAs (Courdavault et al. 2014).

At non-stressful physiological conditions, strictosidine (first MIA engaged in the biosynthetic pathway) concentrations remain low in *C. roseus*. High concentrations of strictosidine may be triggered, for example, after hormonal treatment mimicking the attack of herbivores and/or microorganisms. *Catharanthus* alkaloids and enzymes involved in biosynthetic pathways are compartmentalized, being strictosidine accumulated in vacuoles of epidermal cells. Biosynthesis of strictosidine precursors, however, is restricted to the cytosol of epidermal cells. An ER-anchored P450 secologanin synthase (SLS) and two soluble enzymes, tryptophan decarboxylase (TDC) and loganic acid methyltransferase (LAMT), involved in strictosidine precursor synthesis, are found in the cytosol compartment and operate as homodimers, preventing enzyme passive diffusion into the nucleus. Further strictosidine accumulation in the vacuole occurs via internalization of precursors and strictosidine synthase (STR) activity within this organelle. β-D-Glucosidase (SGD) is the first downstream enzyme after first MIA formation (strictosidine), leading to aglycone biosynthesis and subsequent generation of all other *Catharanthus* MIAs, including the well-known catharanthine, tabersonine, and vindoline. SGD is restricted to the nucleus, and strictosidine exportation from the vacuole must be tightly controlled to avoid accumulation of the aglycone, which is highly reactive and induces strong protein cross-linking. During an herbivore attack, sudden break of substrate (strictosidine) and loss of enzyme (SGD) compartmentalization lead to cellular disruption and massive production of reactive aglycone, readily conferring deterrent/toxic properties to the plant (Courdavault et al. 2014; Fig. 2).

Catharanthine is derived directly from strictosidine, by a currently unknown mechanism, and accumulates in the surface of both below- and aboveground parts of the plant, with almost all content on leaf surfaces. An active transport of catharanthine secretion in wax exudates is mediated by TPT2 [catharanthine transporter pleiotropic drug resistance (PDR) family of ABC transporters], the first characterized MIA transporter. This very specific alkaloid accumulation forces bio-aggressors to face fungicidal and insecticidal properties of catharanthine as the first protection barrier of *C. roseus* (Roepke et al. 2010). The external barrier strength is enhanced by wax exudate enrichment with other active compounds (Courdavault et al. 2014).

One of the most abundant MIAs in *C. roseus* leaves is vindoline, resulting from a six-step modification of tabersonine, and accumulated in laticifers/idioblasts. While only traces of dimeric MIAs are present in plant leaves under physiological conditions, formation of dimeric MIAs, such as vincristine or

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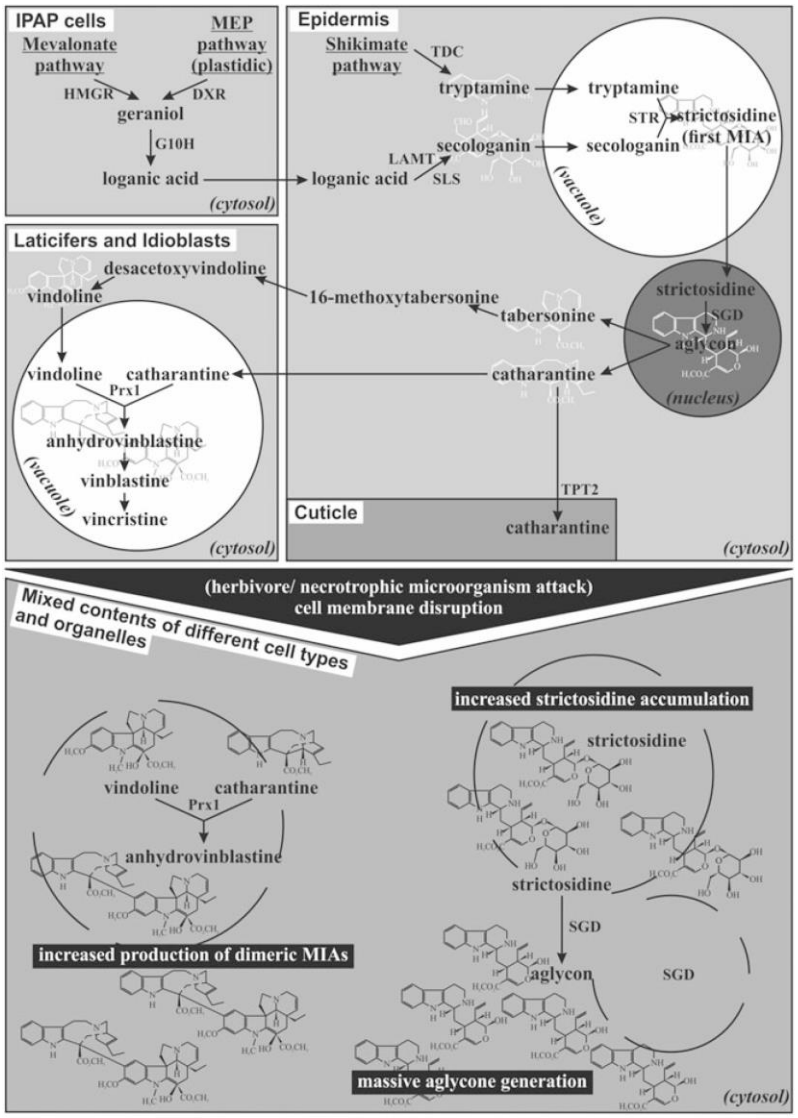

Fig. 2 A simplified version of the main steps of monoterpene indole alkaloid biosynthesis in leaves of *Catharanthus roseus*, indicating the cell types and subcellular compartments involved, and the changes deployed upon herbivory or pathogen attack

anhydrovinblastine, also toxic to bio-aggressors by their microtubule disassembly properties, could result from a mixture of secreted catharanthine with alkaloids released from specialized cells in the presence of a vacuolar class III peroxidase (PRX1) in injured leaves (Courdavault et al. 2014).

MIAs from some South Brazilian *Psychotria* (Rubiaceae) species have also been studied focusing on the influence of environmental factors. Brachycerine, GPV (N,β-D-glucopyranosyl vincosamide), and psychollatine, from the understory species *P. brachyceras*, *P. leiocarpa*, and *P. umbellata*, respectively, are the major alkaloids in these plants. Some common features of these three alkaloids in adult plants include shoot-specific accumulation, high levels of alkaloids in leaves (0.1 % DW to 4 % DW – dry weight), higher content of alkaloids in inflorescences and lower in fruits, broad and strong antioxidant properties, and relatively simpler structures, comparable to that of strictosidine, including the retention of

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one or two glucose residues. GPV seems to be derived directly from strictosidine, whereas brachycerine and psychollatine accumulation may depend on an STR-like enzyme. The monoterpene moiety precursors are not secologanin, but likely epiloganin for brachycerine and a geniposide-derived terpene for psychollatine (Pasquali et al. 2006).

Whereas the leaf concentrations of GPV in *P. leiocarpa* and psychollatine in *P. umbellata* remain constitutively high, not changing under the effect of various stress factors, the concentration of the alkaloid brachycerine (of *P. brachyceras*) is highly responsive to various signals. These include JA application, mechanical wounding, drought, heavy metal exposure, high temperature, and UV-B radiation. The latter stimulus increases its content by approximately 10 times compared to basal levels. Accumulation of brachycerine occurs only in the damaged site, not becoming systemic to the whole plant (Matsuura and Fett-Neto 2013). For seedlings, light presence and developmental stage affect GPV levels, indicating a regulation of alkaloid dynamics by photoautotrophic activity and developmental regulation (higher contents in older seedlings). At least for psychollatine, a tight regulation mechanism involving compartmentalization in specialized cells and organelles may be required; the alkaloid is absent in undifferentiated cell cultures or in rhizogenic calli, even if greened under light, but is accumulated again as soon as embryos start to regenerate from the calli (Paranhos et al. 2005). In *P. brachyceras* leaves, epidermis analysis revealed enrichment of brachycerine in epidermal cells, also indicating specialized compartmentalization.

The main reason for these *Psychotria* MIAs' accumulation seems not directly related to protection against herbivores, as shown by the lack of deterrence or other toxic effects in tests with both specialist and generalist herbivores. Allelopathic effects of the alkaloids in target plant species were also lacking. Because of their efficient antioxidant properties against most types of reactive oxygen species, they may assist in general oxidative stress detoxification (Matsuura and Fett-Neto 2013). *P. brachyceras* and *P. leiocarpa* are resistant to acute UV-B doses, and this protection is mainly caused by brachycerine and GPV presence, which has been shown to improve UV-B tolerance in UV-B-sensitive plants, being this protection linked to the antioxidant properties of these alkaloids (Matsuura and Fett-Neto 2013; Porto et al. 2014). Similarly, the indole alkaloid pityriacitrin (Machowinski et al. 2006) and benzylisoquinoline alkaloid sanguinarine (Ahsan et al. 2007) have been shown to be very efficient in UV-B protection when applied on skin.

P. somniferum (Papaveraceae), one of oldest medicinal plants in the world, is considered the model plant in the study of benzylisoquinoline alkaloids (BIAs) and remains as the only commercial source of morphine and codeine. Other important alkaloids produced by *P. somniferum* include papaverine, noscapine, and sanguinarine. BIAs are also found in plants from the order Ranunculales, in particular Ranunculaceae, Berberidaceae, and Menispermaceae families. BIA production in *P. somniferum* occurs in sieve elements and specialized laticifers; in the latter structures, most BIAs are stored. Phloem tissues are not always involved in BIA biosynthesis, as seen in *Thalictrum flavum* (Ranunculaceae). Phthalide isoquinoline, morphinan, and benzylisoquinoline alkaloids, such as noscapine and papaverine, are major compounds in latex from aerial parts of the plants, whereas benzophenanthridine alkaloids (e.g., sanguinarine) are predominant in roots (Hagel and Facchini 2013; Beaudoin and Facchini 2014).

Defensive roles for BIAs include anti-herbivory, antifungal, and antibacterial properties; in addition to the presence of defensive compounds in the latex, its glue-like consistency per se seems to act as a defense mechanism against foraging herbivores. Mechanically damaged *P. somniferum* was shown to rapidly increase incorporation of bismorphine into the cell wall, decreasing susceptibility to hydrolysis by pectinases, which are often present in salivary secretion of herbivores and are also produced by fungi (Beaudoin and Facchini 2014).

Nicotiana sp. (Solanaceae) contains high levels of the pyridine alkaloid nicotine, playing a role in protection against insect herbivores. Nicotine biosynthesis occurs in the roots of *Nicotiana* plants and is

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transported via xylem to leaves and other parts of the plant by a multidrug and toxic compound extrusion (MATE) transporter; nicotine is primarily stored in the cell vacuoles of aerial parts. Removal of shoot tips and attack by herbivores quickly increase nicotine levels in *Nicotiana*. Auxins are negative regulators of nicotine accumulation, whereas abscisic acid may have a dual effect. Ethylene response factors (ERFs), which are involved in nicotine level regulation, were identified in *Nicotiana* and are positively regulated by abscisic acid. Downregulating ARF1, an auxin response factor, increased nicotine basal concentration, whereas silencing of *NbERF1* had the opposite effect on both basal and stimulated nicotine accumulation (Todd et al. 2010; Wang and Bennetzen 2015). JA is well known as a positive regulator of nicotine biosynthesis, via activation of MYC2-like bHLH (basic helix-loop-helix) transcription factors (TFs) in *Nicotiana*, which directly regulate alkaloid production by transactivating alkaloid biosynthetic genes bearing G-boxes in their promoters. JA also indirectly regulates nicotine accumulation by activating the production of B-locus ERF transcription factors, which bind to GCC-boxes in promoters of genes encoding biosynthetic enzymes. The F-box protein COI1 (coronatine-insensitive protein 1) is an important regulator of JA signaling, acting as a receptor, which interacts with JA-Ile, (+)-7-iso-Jasmonoyl-L-isoleucine, targeting the transcriptional repressor protein JAZ (jasmonic acid ZIM domain) for degradation in the proteasome, so that MYC2 TFs are released for action (Dewey and Xie 2014). The role of JA and JA-Ile has also been established in the regulation of MIA production in *C. roseus* through the control of TFs such as the ORCA family, involved in the coordinated transactivation of biosynthetic genes in both primary and secondary metabolism (Wasternack and Hause 2013).

Signaling for Alkaloid Biosynthesis in Plants

The success of plants is significantly based on their ability to rapidly recognize specific environmental signals and biotic attacks and promote signal transduction pathways that lead to the biosynthesis of defensive compounds (Okada et al. 2015). Recognition of herbivores and pathogens in plants can be conceptually separated in three distinct responses, which are recognition of oviposition, leading to herbivory-induced immunity (HTI), perception of damage or herbivore via DAMPs (damage-associated molecular patterns) and HAMPs (herbivore-associated molecular patterns) leading to HTI, and mechanical wounding, generating wound-induced resistance (WIR). JA is the most important signaling molecule in plant defense triggered by herbivores and mechanical wounding, leading to elicitation of several metabolites including alkaloids. JA biosynthesis can be regulated by different ways. Control of JA biosynthesis is done by a positive feedback loop and also specificity of tissue and substrate availability. Moreover, the synthesis of JA is regulated by different branches in the upstream lipoxygenase (LOX) pathway; hydroperoxide lyase (HPL) branch is known for oxylipins, both volatiles (green leaf volatiles – GLVs) and nonvolatiles, which are leaf aldehydes and alcohols involved in plant defense against herbivores and long-distance signaling (Wasternack and Hause 2013).

GLVs are a class of volatile organic compounds (VOCs) and are involved in indirect plant protection by signaling to distal parts of the attacked plant and to neighbor plants the incoming danger. GLVs also attract carnivorous arthropods, as well documented for lima beans (Kautz et al. 2014). At belowground, VOCs are also important players in plant defense; the quality of VOCs emitted from roots is altered when the hybrid *Festuca pratensis* × *Lolium perenne* is in symbiosis with the fungus *Neotyphodium uncina* colonizing aerial parts, enhancing production of insect-toxic alkaloids in the whole plant (Rostás et al. 2015).

Another regulation point of JA biosynthesis occurs via Ca^{2+} and MAPK cascades. During JA accumulation induced by herbivory or wounding in *Nicotiana attenuata*, activation of wound-induced protein kinase (WIPK) occurs in the wound site, activating JA biosynthesis. The Ca^{2+}-dependent protein

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kinases CDPK4 and CDPK5 negatively regulate the process. In response to many biotic and abiotic conditions, Ca^{2+} acts as a second messenger; Ca^{2+} is involved in modulating the response against herbivores through a calmodulin-like protein CLM42, which acts in decreasing COI1-mediated JA sensitivity downstream of damage-induced Ca^{2+} increase. Calcium may also increase resistance to necrotrophic pathogens and regulate SA levels (Wasternack and Hause 2013).

The mechanisms of perception of the environment and transduction of these external signals to activate alkaloid biosynthetic pathways are of great importance to define and exploit the ecological roles of these compounds, as well as to define strategies to increase their production. Among the strategies to produce alkaloids, plant cultivation and management techniques to improve the content of the metabolite of interest prior to extraction are important tools. Several bioactive plant alkaloids are very complex molecules of difficult and expensive chemical syntheses. Plant cell cultures, both in suspension and immobilized, may also represent a very interesting source of bioactive alkaloids due to the features of cleaner extraction, production independent of weather conditions, and amenability to scale up. Organ cultures are another interesting strategy, particularly roots, which retain a good degree of cellular differentiation, sometimes required for alkaloid biosynthesis, and can be cultivated in large scale (Pasquali et al. 2006). *R. serpentina* hairy root cultures, induced by *Agrobacterium rhizogenes*, are a promising system for production of alkaloids and are considered an experimental model for metabolic engineering in plants due to biochemical stability, fast growth rates, and easy manipulation (Yang and Stöckigt 2010). Hairy roots of *R. serpentina* can yield twice as much of the medicinal alkaloid reserpine compared to field-grown plants (Mehrotra et al. 2015).

For larger scale production of complex plant alkaloids, molecular strategies would be a preferred tool. Some key points for genetic manipulation involve the knowledge of plant interspecific diversity, elucidation of biosynthetic pathways, technology for gene knockout, silencing or overexpression of key points of biosynthetic routes, or master regulator TFs, both with constitutive or inducible promoters in plants or cell cultures (Yang and Stöckigt 2010; Nascimento and Fett-Neto 2010). Major research efforts have also been focused on introducing plant alkaloid biosynthetic pathways in bacteria or yeasts in order to take advantage of the numerous biochemical engineering tools for large-scale production of metabolites in microorganisms (Hagel and Facchini 2013).

Conclusions and Future Directions

Alkaloids are a large and diverse group carrying a broad range of biological activities of great importance to plants, animals, and humans, with highly significant pharmaceutical properties. The study of alkaloid biosynthesis by dissecting the key enzymes of high metabolic flux control, TFs, their encoding genes, and the regulatory controls of metabolism can be used to improve alkaloid production. It may also provide a better understanding of the complex ecological roles of alkaloids and foster the discovery of new drugs or toxins. On the alkaloid supply front, it appears that future efforts will focus on the use of synthetic biology approaches to engineer metabolic pathways leading to plant alkaloids in microorganisms.

Often alkaloids once viewed as “villains,” due to their high toxicity, may be reassessed as holding the cues for combating specific diseases. New emerging ecological roles for alkaloids are also surfacing, such as their activity as antioxidants and general stress protectants, for example, in the case of *Psychotria* MIAs. The primary functions of alkaloids may differ in the various plant species, and their metabolic profiles can be linked to specific environmental factors and developmental signals, often conferring a clear adaptive value. Such dynamic profiles of plant alkaloid metabolism and accumulation are key factors to be considered regarding toxicity to other organisms or bioactive metabolite production for therapeutic purposes.

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The bioactive monoterpene indole alkaloid N,*β*-D-glucopyranosyl vincosamide is regulated by irradiance quality and development in *Psychotria leiocarpa*

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The bioactive monoterpene indole alkaloid *N*,β-D-glucopyranosyl vincosamide is regulated by irradiance quality and development in *Psychotria leiocarpa*

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ABSTRACT

Leaves of *Psychotria leiocarpa* accumulate a major shoot-specific indole alkaloid, *N*,β-D-glucopyranosyl vincosamide (GPV), which has antioxidant, but no apparent antifeedant or allelopathic activity. The species is tolerant to high doses of UV-B, possibly due to constitutive GPV accumulation in leaves. In seedlings, GPV accumulation is induced by white light in a photosynthesis independent fashion. To better understand the regulation of the *in planta* GPV pool, detailed alkaloid profiles were examined. Light shift tests confirmed that GPV accumulation is light dependent. GPV was tightly regulated in its organ distribution, with highest concentrations in reproductive structures. Far-red and blue wavelengths promoted GPV accumulation without clear correlation with carbohydrate or protein concentrations or dry biomass in seedlings, thus indicating direct effects of light. Since light conditions are often associated with higher oxidative stress, light-induced accumulation of GPV may contribute to maintain redox balance. *In vitro*, GPV was an overall more effective antioxidant compared to closely related alkaloids. Indirect evidence of potential antioxidant properties of GPV *in vivo* was obtained by application of GPV on leaves of UV sensitive species exposed to high doses of UV-B, which significantly improved tolerance to this stress. In contrast to the known constitutive accumulation of GPV in leaves, the alkaloid concentration proved to be highly dynamic, changing during development, reproductive organogenesis and seedling irradiance treatments. Data further support a role for GPV as an oxidative stress protectant and provide a means to improve alkaloid yields in plant biomass for pharmacological applications.

1. Introduction

Plant alkaloid metabolism is often influenced by development and environmental factors, including light quantity and quality (Vazquez-Flota and De Luca, 1998; Liu et al., 2015). *N*,β-D-glucopyranosyl vincosamide (GPV) is a major shoot specific *N*-glycosylated monoterpene indole alkaloid (MIA) accumulating in *Psychotria leiocarpa* (Henriques et al., 2004). GPV has ROS quenching activity, which may protect the plant against oxidative burst caused by stressful conditions, possibly contributing to the significant tolerance of the species to acute high dose UV-B treatment (Matsuura and Fett-Neto, 2013). In vegetative adult plants, GPV content is relatively high and stable; alkaloid steady-state does not seem to respond to unfavorable environmental conditions such as high UV-B or mechanical wounding. Besides, no antifeedant (Matsuura and Fett-Neto, 2013) or allelopathic effects (Correa et al., 2008) have been observed for GPV. In contrast, GPV accumulation in etiolated *versus* photomorphogenic seedlings is higher in the latter, apparently without a direct involvement of photosynthetic energy supply (Henriques et al., 2004).

Abbreviations: CRY, cryptochrome; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DW, dry weight; FW, fresh weight; GPV, *N*,β-D-glucopyranosyl vincosamide; HPLC, high performance liquid chromatography; MIA, monoterpene indole alkaloid; NBT, nitro blue tetrazolium; PAR, photosynthetically active radiation; PHYA, phytochrome A; PHYB, phytochrome B; ROS, reactive oxygen species; TFA, trifluoroacetic acid; UV, ultraviolet radiation; UV-A, ultraviolet A radiation; UV-B, ultraviolet B radiation; VLFR, very low fluence response.

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P. leiocarpa is highly abundant in its habitat, the understorey of the Atlantic Forest biome, distinguished from unrelated plants by having small white flowers and purple-blue berries when mature. *Psychotria* is the largest genus in Rubiaceae and since conspicuous distinguishing morphological features are not common among its species, indole alkaloids constitute an important tool in chemotaxonomy, given their high diversification (Van de Santos et al., 2001). Although present in high concentrations in some plants (*e.g.* psychollatine from *Psychotria umbellata* reaching 4% DW in inflorescences), the main role of MIAs in these species is not clear, but seems to involve the metabolic redox balance in relation to several environmental stresses (Matsuura et al., 2014).

In spite of being an understorey tree, *P. leiocarpa* is surprisingly tolerant to acute UV-B stress, which coupled to GPV *in vitro* antioxidant properties (Matsuura and Fett-Neto, 2013), suggests a role for the alkaloid in this phenotype. Detailed understanding of GPV metabolism in response to light and developmental signals may help not only to elucidate its main *in planta* role, but also allow the establishment of optimal protocols for harvesting GPV-rich biomass or eliciting its accumulation prior to extraction and isolation, with a view towards pharmaceutical uses of this alkaloid.

Brachycerine, a closely related leaf alkaloid from *Psychotria brachyceras* that has antioxidant and antimutagenic properties (Nascimento et al., 2007), is induced by approximately 10-fold compared to its basal level upon acute UV-B exposure (Gregianini et al., 2003), involving early changes at mRNA level (Nascimento et al., 2013). Unlike brachycerine and similar to GPV, psychollatine (another related alkaloid with similar properties from *P. umbellata*) is not induced by acute UV-B exposure (Fragoso et al., 2008; Paranhos et al., 2009). Therefore, it appears that GPV has a dual profile being induced by light in seedlings and remaining at stable concentrations upon high doses of UV-B exposure in leaves of adult cuttings.

To further understand the details of irradiance and developmental control of GPV accumulation, herein we characterize the influence of light shifts and irradiance quality on GPV biosynthesis in photomorphogenic and etiolated seedlings and the dynamics of GPV accumulation during reproductive growth. A detailed analysis was conducted on the accumulation dynamics of GPV during reproductive growth of adult plants and in different tissues of developing flowers and fruit. The structural basis of antioxidant potential in GPV was studied in an *in vitro* comparative test with similar indole alkaloids from closely related *Psychotria* species and with well-known antioxidants. The *in vivo* antioxidant and protective effect of GPV was indirectly examined by application of the alkaloid on leaves of two UV-B sensitive species (*Phaseolus vulgaris* and *Psychotria carthagenensis*) and evaluation of their tolerance to high doses of UV-B. Taking into account the overall results, potential implications of alkaloid dynamics for GPV *in planta* functions and improved alkaloid yields for pharmaceutical evaluation are discussed.

2. Materials and methods

2.1. Alkaloid isolation and GPV analysis

N,β-D-glucopyranosyl vincosamide (GPV), brachycerine and psychollatine were purified from *P. leiocarpa* Cham. & Schltdl., *P. brachyceras* Mull. Arg. and *P. umbellata* Vell. leaves, respectively (harvest license by Federal Authority Sisbio/ICMBio 32855-1; authentication code: 58482685), following previously described methods with minor modifications (Henriques et al., 2004; Both et al., 2002; Kerber et al., 2001). High Performance Liquid Chromatography (HPLC) was used to evaluate compound purity in comparison to authentic alkaloids. For GPV analysis, methanolic extracts from samples were analyzed by HPLC as previously described (Henriques et al., 2004). Shortly, fresh tissue samples (250 mg each) were macerated in liquid nitrogen and 1 mL of cold methanol (HPLC grade, Merck) was added; the extract was then sonicated for 30 min at 4 °C and the extract centrifuged at 13,000*g* at 4 °C for 15 min. Supernatant was recovered and analyzed by HPLC. Extracted dry weights (DW) were obtained after drying pellets at 60 °C until constant weight. The samples were analyzed in a Thermo Scientific Surveyor HPLC with a C18 reverse phase column equipped with respective guard column (Shimadzu) using linear gradient (1 mL min^{-1} flow), starting with water: methanol (60:40), and ending with methanol. Trifluoroacetic acid (TFA) (Sigma) was added to a final concentration 0.05% in both eluents. An external standard curve was prepared with authentic GPV. Content of GPV was expressed on a dry weight basis. Voucher specimens are deposited at the ICN herbarium at UFRGS (138157-*P. leiocarpa*; 7899-*P. brachyceras*; and 98869-*P. umbellata*).

2.2. Psychotria leiocarpa *seed germination*

Fruits were harvested from field grown *P. leiocarpa* plants (Morro Santana, Porto Alegre, Brazil). After pulp extraction, seeds were stored at 10 °C for two weeks before surface disinfection with 70% ethanol (v/v) (1 min) and 1.5% NaClO (v/v) (10 min). For aseptic germination, seeds were maintained in 0.1× MS (Murashige and Skoog, 1962) nutrient medium salts, pH 5.8, containing 0.6% agar with or without 1.5% sucrose (w/v), and grown under aseptic conditions at 26 ± 2° C in light [35 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), 16 h day^{-1} photoperiod] or dark. For non-aseptic germination, seeds were planted in an autoclaved substrate composed of top commercial soil and vermiculite (1:1, v/v) and kept in a growth chamber (16 h day^{-1} photoperiod, 60 μmol m^{-2} s^{-1} PAR and 28 ± 2° C).

2.3. Dark-light transitions and GPV accumulation

Seedlings from *P. leiocarpa* containing six to ten leaves grown in aseptic conditions without sucrose were used to investigate light influence on GPV biosynthesis. A group of light grown seedlings was transferred to dark (at least 5 seedlings per sample, and at least 5 samples per treatment) and a group of dark grown seedlings was transferred to light (same numbers as for the light to dark group). Both groups were kept in the respective condition for 14 days. For GPV analysis, aerial parts of seedlings were harvested after 7 and 14 days of the light shift, immediately frozen in liquid nitrogen, and stored at −20 °C until analysis. Transitions from light to dark in light grown seedlings and dark to light in etiolated seedlings (25 days in darkness after germination) were also carried out to evaluate the role of sucrose presence or absence in growth media. Aerial parts of seedlings were harvested after 14 days of transition.

2.4. Light quality effects on concentrations of GPV, proteins and carbohydrates, and on dry biomass of seedlings

To identify the possible photoreceptor system involved in GPV light regulation, different wavelength enrichment assays were performed. Seedlings containing six to ten leaves from non-aseptic germination were exposed to white light filtered through different cellophane sheets (transparent, blue, red or green) for light enrichment in different wavelengths (control, blue, red or far-red respectively) (Ruedell et al., 2013). The absorbance spectra were obtained by wavelength scans in a Cintra 5 spectrophotometer (GBC, Victoria, Australia) (see Supplementary Fig. S1 in the online version at DOI: 10.1016/j.indcrop.2016.03.050). Treatment PARs were normalized at 30 μmol m^{-2} s^{-1} in the first assay and 90 μmol m^{-2} s^{-1} in the second, for which previously etiolated plants were used (after 55 days in dark). Seedlings were harvested

after 5, 10, 15, 20 and 25 days of treatment in the first assay, and after 7, 17 and 27 days of treatment in the second assay. Samples were evaluated for GPV, soluble protein and soluble sugar contents following previously established protocols (Henriques et al., 2004; Bradford 1976; Ghosh and Majumdar, 2003, respectively). Dry biomass of seedlings was obtained by incubation in an oven at 60 °C until constant weight.

2.5. GPV distribution in leaves of different ages and in reproductive organs

Two vegetative stages (young and fully expanded adult leaves) and four reproductive stages (inflorescence with floral buds, inflorescence with open white petal flowers, young green and mature purple fruits) of *P. leiocarpa* tissues were analyzed for GPV content in two different populations. In open flowers and mature fruit, different parts were analyzed after mechanical separation (flower outer and inner whorls and fruit peel or epicarp, pulp, and seed).

2.6. Comparative in vitro antioxidant assay

In vitro quenching activities against superoxide and singlet oxygen were performed essentially as previously reported assays (Gregianini et al., 2003; Paranhos et al., 2009; Matsuura and Fett-Neto, 2013). Solvent only (water-methanol, 1:1, v:v), Trolox™ and/or rutin (positive controls, same concentration as *Psychotria* alkaloids) or alkaloids 0.5 mM (superoxide assay) or 1 mM (singlet oxygen assay) (GPV, brachycerine and psychollatine) were added to the medium before reaction. All procedures were performed under dim light. Shortly, for singlet oxygen assay, 0.8 mM rubrene chloroform solution was evaluated for color decay (absorbance at 440 nm) after white light exposure (22 J cm^{-2}). Superoxide anion quenching activity was measured by Nitro Blue Tetrazolium (NBT) reduction; a solution containing 3 μM riboflavin, 10 mM methionine, and 0.1 mM NBT in phosphate buffer 50 mM, pH 7.8, was exposed to white light (350 μmol m^{-2} s^{-1}), and the absorbance at 560 nm of the samples was measured before and after light exposure.

2.7. Evaluation of GPV application on leaves of UV-B sensitive species as a high dose UV-B protectant

Leaf disks (n = 12 per replicate) from *P. vulgaris* L. (1 cm diameter) (seeds obtained at a local market and plants cultivated for 2 weeks in a growth room) and *P. carthagenensis* Jacq. (2 cm diameter) (voucher specimen 7901 at ICN herbarium- UFRGS) were treated with methanol on the adaxial surface (control samples, lacking alkaloid) or with a methanol solution containing GPV at 10 mM or 20 mM (this last one only for *P. carthagenensis*), similar to concentrations found in *P. leiocarpa* leaves. After methanol evaporation, samples were exposed to acute UV-B radiation [130.90 kJ m^{-2} day^{-1}, 47.14 kJ m^{-2} day^{-1} biologically effective radiation (Caldwell, 1971)] for 48 h (*P. vulgaris*) or 96 h (*P. carthagenensis*) (16 h day^{-1}, 25 ± 2° C); a glass plate (5 mm thick) was placed above the disks to filter out UV-B and most UV-A wavelengths in a control treatment. In all treatments, white light irradiance was available at 40 μmol m^{-2} s^{-1} PAR. Chlorophyll contents from the samples were evaluated by binary mixture absorbance analysis in spectrophotometer (Ross, 1974). HPLC analyses of *P. carthagenensis* samples containing GPV were as described by Henriques et al. (2004).

2.8. Statistical analysis and experimental layout

Experiments followed a totally randomized layout. Results were checked for normal distribution and analyzed by ANOVA followed by Duncan, $P \leq 0.05$, or non-parametric Welch's ANOVA followed by Dunnett's T3 when necessary, using statistics package SPSS 17.0. All assays described had treatments performed in biological triplicates or quadruplicates, and each assay was independently repeated.

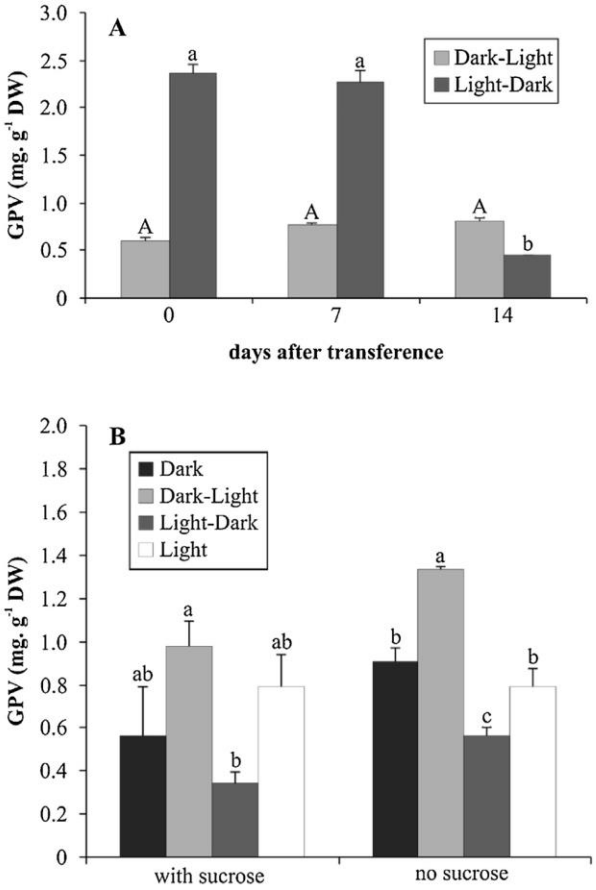

Fig. 1. (A) GPV content (mg g^{-1} dry weight) of plantlets growing without sucrose, cultivated in continuous dark before transfer to light (Dark-Light) or cultivated in light (16 h day^{-1} photoperiod) before transfer to dark (Light-Dark), after 7 and 14 days of transfer. Statistical tests were run separately for each group (Dark-Light and Light-Dark). (B) GPV content (mg g^{-1} dry weight) of plantlets growing in presence or absence of sucrose, in continuous light or dark, and cultivated in continuous dark before transfer to light (Dark-Light) or cultivated in light (16 h day^{-1} photoperiod) before transfer to dark (Light-Dark), 14 days after transference. Bars within group sharing same letters do not differ significantly by a Duncan test ($P \leq 0.05$). Lines on top of bars are standard errors of the means.

3. Results

3.1. Light-dark shifts and seedling GPV

Dark-light and light-dark transition assays showed light to be a necessary condition for GPV accumulation and maintenance at higher levels in seedlings (3 to 4-fold higher content in light-grown samples) (Fig. 1A). Such light dependence was observed in spite of the presence of sucrose in medium, since similarly positive effects of light and negative of dark were evident independent of exogenous sucrose supply (Fig. 1B). After 14 days of a light-dark transfer, GPV content in seedlings decreased from 0.23% DW to 0.045% DW, reaching contents comparable to those of dark-grown seedlings (Fig. 1A). Germination tests indicated a certain degree of positive photoblasty in *P. leiocarpa* seeds; higher germination rates were

Table 1
In vitro antioxidant tests against superoxide anions (%NBT reduction) or singlet oxygen (absorbance decay at 440 nm) comparing psychollatine, brachycerine and GPV (0.5 mM in superoxide assay and 1 mM in singlet oxygen assay); Trolox™ and rutin (when indicated) were used as positive controls (0.5 mM in superoxide assay and 1 mM in singlet oxygen assay). Values not sharing a letter within the same assay are significantly different by a Duncan test ($P \leq 0.01$). IC 50 = concentration to inhibit 50% of the transformations. $K_q{}^1O_2$ = singlet quenching rate.

Compound	% NBT reduction	IC 50	Decay (Δ absorbance)	$K_q{}^1O_2$
Solvent only	90.126 ± 0.291a	–	1.922 ± 0.150a	–
Rutin	10.544 ± 3.596cd	0.022	–	–
Trolox™	15.684 ± 1.136c	0.074	1.398 ± 0.114c	1.2×10^7
Psychollatine	27.676 ± 2.291b	0.064	1.555 ± 0.131bc	8.9×10^6
Brachycerine	11.063 ± 0.837cd	0.062	1.739 ± 0.092ab	2.5×10^6
GPV	6.929 ± 0.168d	0.047	1.507 ± 0.212bc	7.2×10^6

recorded in seeds exposed to light (45%) compared to those germinated in darkness (10%). Due to low germination rates in the dark, a pre-treatment to etiolate light-germinated seedlings was necessary (25 days in darkness for etiolation). Etiolated seedlings transferred to light maintained alkaloid content similar to initial levels after 14 days (0.07% DW) (Fig. 1A).

3.2. Light quality and GPV

In the light quality assays, overall trends for decreases in protein and carbohydrate concentrations were apparent in photomorphogenic plants and these were associated with GPV increase (Fig. 2A–C). The highest contents of GPV matched the lowest carbohydrate levels after 25 days (Fig. 2A and B). The dry weight of seedlings of the different light conditions along the five time points evaluated did not show statistical differences (data not shown). Etiolated seedlings, control and red light-enriched exposed seedlings did not show significant changes in GPV, protein or sugar contents, whereas far-red and blue light enrichment lead to higher alkaloid accumulation (Fig. 2D–F). As expected, etiolated seedlings had considerably less GPV compared to photomorphogenic plants (Fig. 2A and D).

3.3. GPV distribution in leaves of different ages and in reproductive organs

GPV concentration varied according to specific organs and developmental stages (Fig. 3). Clearly, commitment with the reproductive phase caused a major increase in GPV accumulation. Floral buds and open flowers had the highest contents of the alkaloid. In open flowers, the inner reproductive whorls concentrated most of the GPV (Fig. 3A). In contrast, a marked decrease in GPV content was observed for both green and mature fruit (Fig. 3B and C). In fruit, the outer tissues of the epicarp (peel) had higher concentration of GPV, whereas the pulp and seed had much lower amounts (Fig. 3B and C). These results were consistent in two different *P. leiocarpa* populations (data not shown).

3.4. Comparative antioxidant capacity of GPV

To test the relative capacity of GPV to act as antioxidant, a direct *in vitro* comparison was carried out against related alkaloids and well known non-enzymatic antioxidants. Comparisons among the quenching activities of related *Psychotria* MIAs (brachycerine, GPV and psychollatine) revealed some apparent structure-activity correlations. In superoxide anion quenching assay, increase in absorbance intensity at 560 nm indicates higher NBT reduction rate and consequently lower quenching activity for superoxide anions. In the negative control, 90% of NBT reduction was reached after 8 min of light exposure (Table 1). GPV and brachycerine were more efficient (lower than 12% NBT reduction) in comparison to psychollatine (27% NBT reduction), yielding results comparable to those of the positive controls Trolox™ (15% NBT reduction) and the flavonoid rutin (10% NBT reduction) (Table 1). The IC50 of GPV was approximately twice that of rutin, and 60% that of Trolox®, the positive controls. In singlet oxygen assay, marked color intensity decay at 440 nm reflects poor singlet oxygen quenching activity. As expected, the negative control had the highest decay among tested samples. GPV and psychollatine showed similar quenching activity after 4 min of light exposure ($K_q{}^1O_2$ values $7.2E^6$ and $8.95E^6$, respectively), whereas brachycerine yielded weaker activity ($K_q{}^1O_2$ value of $2.5E^6$) (Table 1). Trolox™ had the highest activity (lowest decay, $K_q{}^1O_2$ $1.2E^7$). Overall, GPV proved to be the most efficient antioxidant among these three alkaloids (see Supplementary Fig. S2 in the online version at DOI: 10.1016/j.indcrop.2016.03.050).

3.5. UV-B tolerance and GPV

To test whether GPV antioxidant properties could operate *in vivo*, an acute and intense UV-B exposure assay was performed with or without prior application of GPV on leaf disks of two UV-B sensitive plants lacking the alkaloid, *P. vulgaris* and *P. carthagenensis*. GPV conferred to these plants tolerance to UV-B as indicated by maintenance of chlorophyll content after 48 h (*P. vulgaris*) or 96 h (*P. carthagenesis*) of exposure (Fig. 4a). Additionally, GPV recovered from leaf disks directly exposed to UV-B remained stable after 96 h (extracted from *P. carthagenensis* leaf-disks surface), as visualized in HPLC profiles (Fig. 4b). The ratio of chlorophyll a/b was less reduced by UV-B exposure with GPV treatment in *P. vulgaris* (38% reduction with GPV *versus* 58% in control), whereas it was maintained in *P. carthagenensis* (see Supplementary Fig. S3 in the online version at DOI: 10.1016/j.indcrop.2016.03.050).

4. Discussion

Different enzymes of MIA biosynthesis may be distributed among several subcellular compartments, including endoplasmic reticulum, cytosol, vacuole, and chloroplast (Facchini and DeLuca, 2008). In etiolated seedlings, the light exposure period was probably not sufficient for the required re-establishment of the cellular and enzymatic machinery needed for GPV biosynthesis. The higher levels of GPV in treatments devoid of sucrose may be a consequence of increased photosynthetic carbon metabolism and the higher contribution of specific biosynthetic intermediates, such as plastid biosynthesis of terpene molecules (*e.g.* secologanin) in photosynthetically active plants (Memelink et al., 2001; Henriques et al., 2004). Such plastid terpene production is likely less active in plants that are partially repressed in photosynthesis by the presence of exogenous carbohydrates (Sheen et al., 1999). In the presence of sucrose, overall GPV content in seedlings was slightly lower compared to absence of sucrose, and etiolated seedlings had GPV contents similar to those of photomorphogenic seedlings. Therefore, the dark pre-treatment could have been too short or perhaps brief exposures to green light during plant etiolation evaluation could suffice to cause some GPV induction. This could characterize the involvement of a reaction to light of the VLFR type (Li et al., 2011).

The light quality assays underlined the positive impact of light on GPV accumulation. In the light quality treatments of etiolated plants, only a few differences were observed in primary metabolism based on the evaluated parameters, suggesting certain homogeneity of response. Comparing time course graphs among photomorphogenic plants, independent of light quality or seedling source, GPV level increases with time (Fig. 2), being positively regulated by ontogeny, in good agreement with previously reported

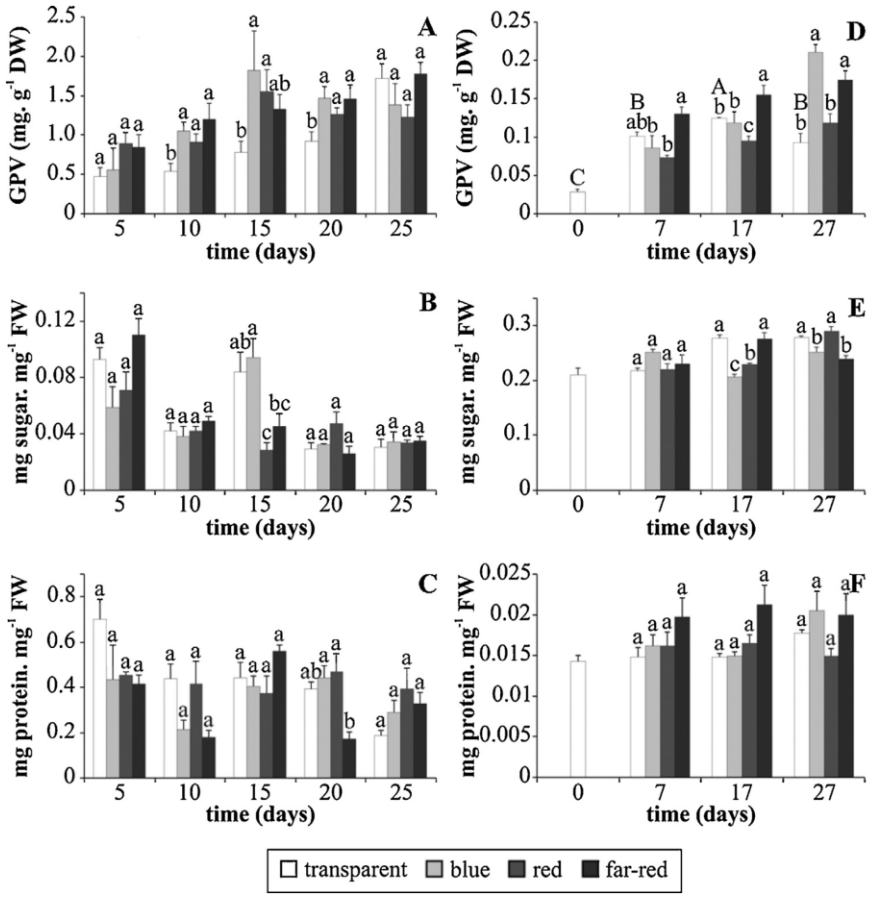

Fig. 2. Light quality enrichment effects on contents of GPV (A and D), total soluble hexoses (B and E) and protein (C and F) of *P. leiocarpa* plantlets; lines on top of bars indicate standard errors of the means. Photomorphogenic plants (30 μmol m^{-2} s^{-1} PAR) (A–C) and pre-etiolated plants (90 μmol m^{-2} s^{-1} PAR) (D–F); white bars: post etiolation control. Bars within group sharing same letters do not differ significantly by a Duncan test ($P \leq 0.05$) or Welchís ANOVA followed by Dunnettís T3 (C and D); capitalized letters correspond to comparison among control condition at each time sampled.

data (Henriques et al., 2004). Positive induction effects of red light and repression by far-red light have been reported for several metabolites (Beegs et al., 1986; Vazquez-Flota and De Luca, 1998; Yamaura et al., 1991; Schofield and Paliyath, 2005). However, it is not always the case that red light stimulates secondary metabolite production. Cinchonine, a pharmacologically relevant naphthoquinone, has its biosynthesis highly inhibited by blue or white light and partially inhibited by red light when compared to dark (Liu et al., 2006). Production of camptothecin, an antitumoral alkaloid from *Camptotheca acuminata*, is induced by green light (Park et al., 2003), blue and red light (Liu et al., 2015). Nicotine content in tobacco decreases upon receiving far-red light supplementation for 5 min (simulating dusk), concomitant to chlorogenic acid (a soluble phenolic) elicitation (Tso et al., 1970). Far-red light has also been involved in metabolite elicitation, such as saponins in *Panax quinquefolius* (Fournier et al., 2003), whereas red light promoted immunoadjuvant triterpene saponin accumulation in *Quillaja brasiliensis* (Yendo et al., 2015).

GPV inducible behavior specifically in initial developmental stages may be related to a primary investment in growth rather than defense against ROS, whose induction can be triggered under specific unfavorable conditions. It is worth considering that *Catharanthus roseus* and most other model species in MIAs research are heliophilous whereas *P. leiocarpa* and other evaluated *Psychotria* species are understorey plants. Metabolite elicitation by far-red intensity variations may be of adaptive and evolutionary meaning in understorey environments, where far red is a predominant form of incoming light, due to filtering of most red and blue light by the upper canopy. The biosyntheses of typical MIAs, including GPV of *P. leiocarpa*, and those of *Catharanthus* and *Rauwolfia*, require the indole pathway to produce tryptamine, which combines with a terpene moiety derived from plastids (in most cases secologanin), yielding the terpene indole alkaloids. The increase of indole concentration is often promoted by low red: far-red ratio during shade avoidance responses, when growth hormones (*i.e.* auxins) are synthesized through a PIF7-regulated pathway involving PHYB (Li et al., 2012). This indole metabolism increase is a condition that may affect tryptamine pools for GPV production. On the other hand, increased GPV contents in de-etiolating seedlings under relatively low irradiance and a prevalence of far red may suggest that GPV biosynthesis could be regulated at least in part by PHYA (Li et al., 2011).

Blue light enrichment also promoted GPV accumulation in a photosynthesis independent fashion. This could give some support for the involvement of CRYs in modulating GPV biosynthesis. CRYs are known for modulating the expression of several secondary metabolic pathways, particularly those of shikimate derivatives, such as phenylpropanoid, phenolic and flavonoid/anthocyanins (Lopez et al., 2012). The concerted action of both PHYs and CRYs in inducing GPV production cannot be ruled out since these photore-

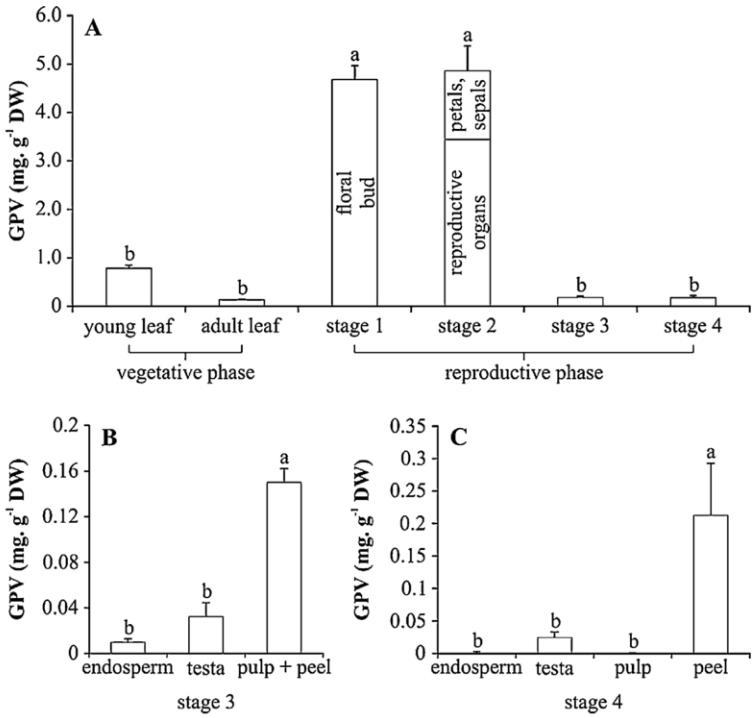

Fig. 3. GPV content (mg g^{-1} dry weight) of different organs of *P. leiocarpa* at different ontogenetic phases. Stage 1 = closed floral bud, stage 2 = open flowers, stage 3 = green fruit, stage 4 = mature fruit. Data are the means of two different populations of trees. Bars indicate standard errors of the means. Different letters indicate significant difference by a Duncan test ($P \leq 0.05$).

ceptors partially overlap their absorption spectra and can cooperate in activating phenylpropanoid metabolism (Hemm et al., 2004).

Organ and tissue distribution of GPV was markedly influenced by reproductive development, similar to what had been previously reported for *P. brachyceras* (Gregianini et al., 2003) and *P. umbellata* (Paranhos et al., 2009). GPV accumulation was higher in floral buds and outer tissues of fruit in agreement with the involvement of light and green tissues in GPV biosynthesis. In general, the recorded contents of GPV in leaves were considerably lower than those reported by Henriques et al. (2004), but very similar to the values determined by Matsuura and Fett-Neto (2013). This discrepancy may reflect biomass harvest from different populations of *P. leiocarpa*, as well as phenology and seasonal changes among distinct years of sampling.

In spite of the lack of overt antiherbivory effects, the higher accumulation of antioxidant alkaloid in reproductive tissues may be relevant for tolerance against multiple stresses, both biotic and abiotic (Matsuura et al., 2014). Antioxidant metabolites, including alkaloids, may help plants cope with oxidative stress caused by herbivore-inflicted damage (Porto et al., 2014). The reduction of GPV content upon the transition from flower to fruit and seed may be the combined result of a lower demand for oxidative stress tolerance at this later stage, reduction of photosynthetic capacity due to loss of chlorophyll and shifting of common precursors (*e.g.* chorismate) from indole to flavonoid/anthocyanin metabolism.

GPV accumulation was induced by light in seedlings and its constitutive presence in leaves of adult plants had been tentatively related to UV-B tolerance in *P. leiocarpa* (Matsuura and Fett-Neto, 2013). The application of realistic amounts of GPV on leaves of two UV-B-sensitive species protected PSII upon acute UV-B exposure, as inferred from chlorophyll concentration, and chlorophyll a/b ratio (see Supplementary Fig. S3 in the online version at DOI: 10.1016/j.indcrop.2016.03.050). These data provide support, albeit indirect, for a possible role of GPV as a protection against oxidative stress because of its chemical properties. The fact that applied GPV was not degraded after UV-B treatment indicated that the alkaloid may play a role as a physical quencher of ROS (Gregianini et al., 2003). A direct comparison of GPV antioxidant activity towards singlet oxygen and superoxide with related alkaloids brachycerine and psychollatine, as well as against the positive control Trolox® (and rutin for superoxide), showed that GPV is highly effective in this activity.

UV-B increases cellular hydrogen peroxide levels, and acts in its partial photo-conversion to hydroxyl radicals, often inflicting cellular damage (Czégény et al., 2014). These radicals may also interact with polygalacturonic acid in cell walls leading to extracellular production of superoxide, possibly affecting cell signaling (Pristov et al., 2013). Application of GPV on leaf adaxial surface and its potential epidermis infiltration can mitigate acute UV-B exposure damage. This property may be due to the alkaloid ROS quenching capacity, as well to UV shielding properties due to its absorbance in this spectral range. UV-B protection of sensitive species *P. vulgaris* and *P. carthagenensis* by GPV treatment was most likely due to the alkaloid antioxidant activity, for leaf cuticles effectively attenuate UV-B (Krauss et al., 1997) and, in spite of that, the cuticle components did not confer protection against UV in control samples.

Specific differences in chemical structure may afford changes in antioxidant efficiency of alkaloids. Other *Psychotria* alkaloids, such as brachycerine from *P. brachyceras* and psychollatine from *P. umbellata*, besides structural similarities to GPV, also share efficient antioxidant activity (Gregianini et al., 2003; Nascimento et al., 2007; Fragoso et al., 2008; Paranhos et al., 2009). Antioxidant activity was also reported for *Psychotria* extracts, including *Psychotria nilgirensis* acetone extract (Iniyavan et al., 2012) and ethanolic extracts from *Psychotria pubigera*, *Psychotria ruelliifolia*, *Psychotria suterella*, *Psychotria capitata*, *Psychotria glaziovii*, *P. leiocarpa*, *Psy-*

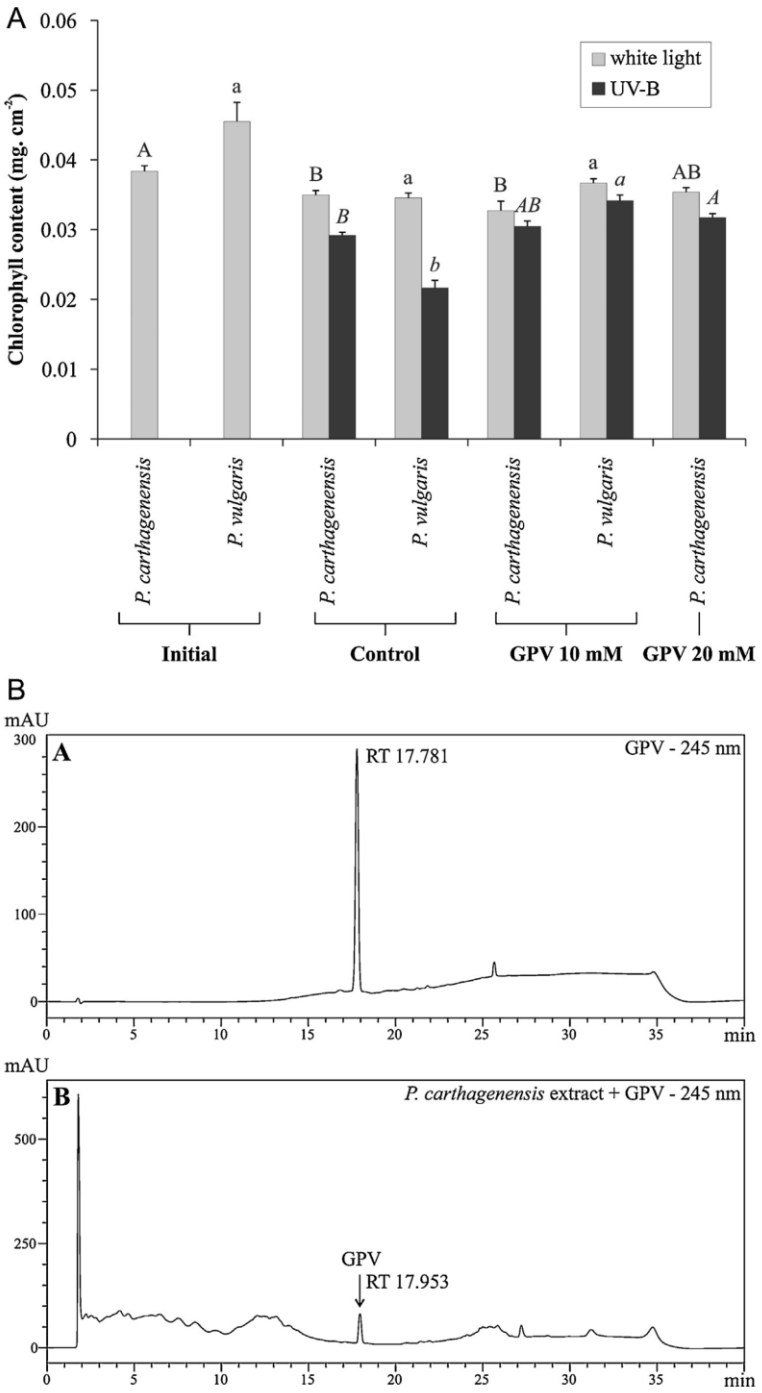

Fig. 4. A. chlorophyll content (mg cm^{-2} leaf dry weight) from leaf disks after 48 h (*Phaseolus vulgaris*) or 96 h (*Psychotria carthagenensis*) of acute UV-B treatment. Bars indicate standard errors of the means. Different letters indicate significant difference by a Duncan test ($P \leq 0.05$); uppercase letters indicate comparison between *P. carthagenensis* treatments and lowercase letters indicate comparison between *P. vulgaris* treatments; statistical tests were run separately for each group (white light and UV-B). B. HPLC profile of authentic GPV solution (A) and GPV-treated *P. carthagenensis* leaf extract after 96 h of UV-B exposure (B). The arrow indicates GPV peak.

chotria nuda, *Psychotria racemosa* and *Psychotria vellosiana* by DPPH assay (Moraes et al., 2011). Chemical defense traits, such as negative effects against the herbivores *Sitophilus zeamais* and/or *Spodoptera frugiperda*, were observed for some *Psychotria* extracts (*Psychotria hoffmannseggiana*, *P. capitata*, *Psychotria goyazensis*, and *P. carthagenensis*) (Tavares et al., 2013; Porto et al., 2014), although the active compounds responsible for observed responses were not determined. Brachycerine, as well as GPV, lacked deterrent activity in different assays (Matsuura and Fett-Neto, 2013; Porto et al., 2014) and was also capable of protecting UV-B sensitive plants against

acute UV-B radiation (D. D. Porto, personal communication), further supporting a correlation between alkaloid structure and functional activity.

Similar structural features are shared among GPV, brachycerine and psychollatine (see Supplementary Fig. S2 in the online version at DOI: 10.1016/j.indcrop.2016.03.050). Some of these shared structural features seem to confer an efficient antioxidant activity (*i.e.* double bonds, secondary or tertiary amines, and glucose residues) and, along with other structural features, may also be linked to the common lack of a role in plant-herbivore and plant–plant direct interactions. In fact, ROS quenching activity seems to be the main physiological advantage provided by accumulation of these alkaloids (Matsuura et al., 2014), protecting light exposed and/or oxidative stress sensitive tissues (leaves, fruit peel and inflorescences). A high correlation between the presence of indole ring and peroxyl radical scavenging activity was found in a study of the electrochemical behavior of various compounds (Estevão et al., 2011). The overall superior antioxidant activity of GPV can be explained by the presence of two tertiary amines and an additional glucose residue in this alkaloid (Paranhos et al., 2009) (see Supplementary Fig. S2 in the online version at DOI: 10.1016/j.indcrop.2016.03.050).

5. Conclusions

The alkaloid GPV is possibly involved in general oxidative stress modulation. GPV showed strong antioxidant activity against superoxide and singlet oxygen, particularly the former, when compared to structurally-related alkaloids. GPV remained stable after 4 days of direct acute UV-B exposure, suggesting physical quenching of ROS.

Light presence proved to be essential for GPV biosynthesis. In early developmental stages of *P. leiocarpa* plants, far-red and blue light enrichment positively affected GPV content, without a consistent correlation with simultaneous impacts on primary metabolism increase (as assessed by carbohydrates, protein content and dry biomass). The stimulatory effect of far-red light on alkaloid accumulation may be a particularly important environmental cue in the understorey.

GPV concentration is higher in reproductive structures and changes along their development. The organ and tissue specific accumulation profiles of the alkaloid, as well as its regulation by light quality, provide guidelines for harvesting plant biomass with higher yields of GPV for pharmacological applications and further bioactivity evaluations.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

A.G.F.N. conceived the experiments. V.F., J.T.P. and H.N.M. helped design the experiments. V.F., J.T.P., H.N.M. and M.R.R. performed the experiments and analyzed the data; H.N.M. drafted the paper and A.G.F.N. finalized it. All authors read and approved the final manuscript.

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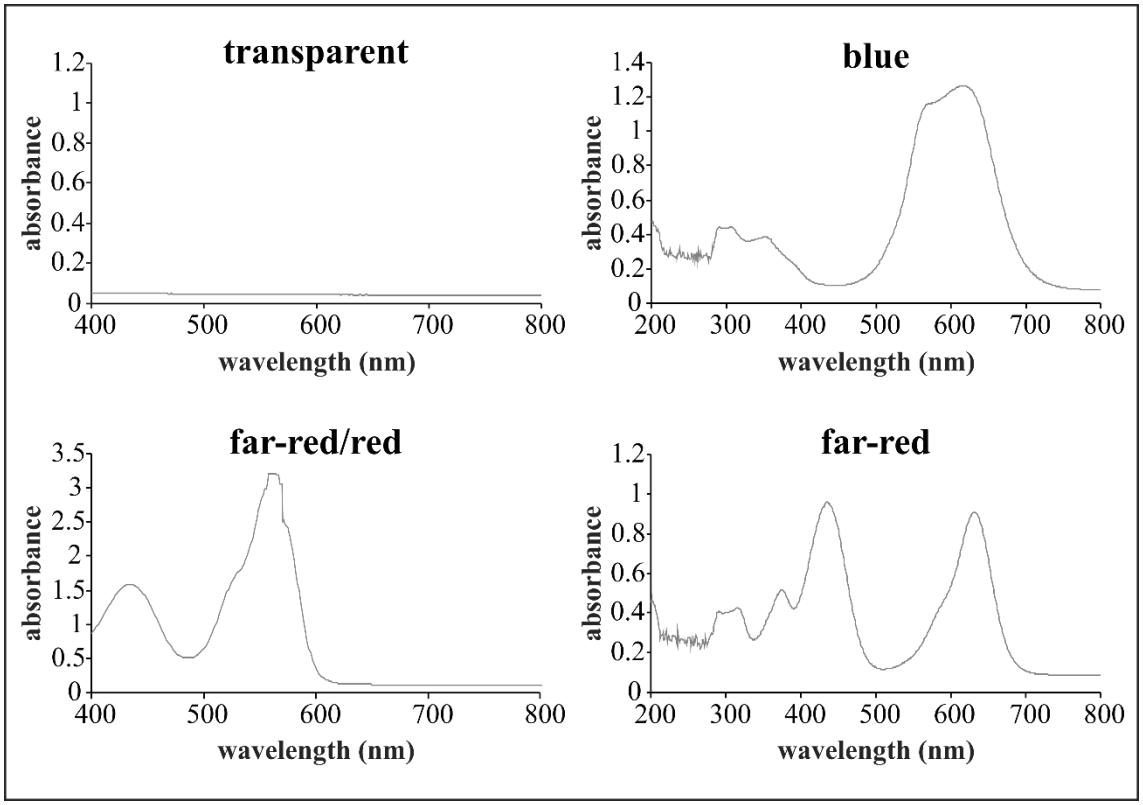
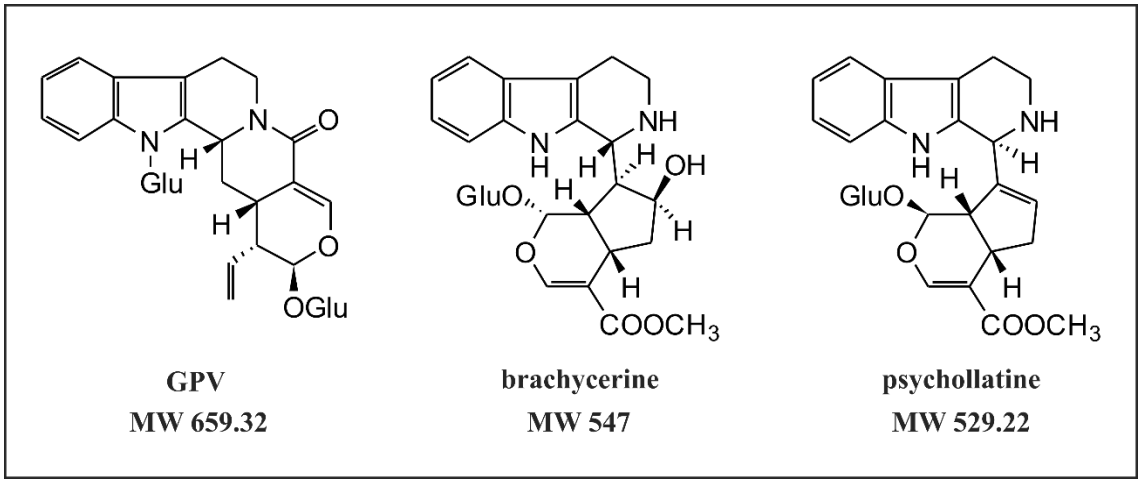
Supplementary Material



Supplementary Figure 1. Absorbance spectrum of filters used in light quality enrichment assay.



Supplementary Figure 2. Structure and molecular weight of *Psychotria* alkaloids used in this study.

Supplementary Figure 3. Chlorophyll a/b ratio from leaf disks after 48 h (*Phaseolus vulgaris*) or 96 h (*Psychotria carthagenensis*) of acute UV-B treatment. Data are expressed in means ± standard errors. Different letters indicate significant difference by a Duncan test ($P \leq 0.05$).

Treatment		**Chlorophyll a/b ratio** ***Phaseolus vulgaris***		**Chlorophyll a/b ratio** ***Psychotria carthagenensis***	
Initial time		2.648 ± 0.022	a	2.266 ± 0.229	a
WL	**control**	1.953 ± 0.147	b	2.491 ± 0.009	a
	GPV 10 mM	1.656 ± 0.039	b	2.459 ± 0.019	b
UV-B	**control**	0.817 ± 0.071	d	2.290 ± 0.011	c
	GPV 10 mM	1.031 ± 0.020	c	2.356 ± 0.017	b

Capítulo III: Artigo de revisão publicado.

Oxidative stress and production of bioactive monoterpene indole alkaloids: biotechnological implications

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REVIEW

Oxidative stress and production of bioactive monoterpene indole alkaloids: biotechnological implications

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Abstract Monoterpene indole alkaloids (MIAs) encompass plant natural products with important pharmacological relevance. They include the antitumoral MIAs found in *Catharanthus roseus* and *Camptotheca acuminata*. The often low yields of bioactive alkaloids in plants has prompted research to identify the factors regulating MIA production. Oxidative stress is a general response associated with biotic and abiotic stresses leading to several secondary responses, including elicitation of MIA production. These changes in secondary metabolism may take place directly or via second messengers, such as Ca^{2+} and reactive oxygen species (ROS). H_2O_2 is the main ROS that participates in MIA biosynthesis. This review analyzes the links between oxidative stress, elicitation of bioactive MIA production and their potential roles in antioxidant defense, as well as exploring the implications to developing biotechnological strategies relevant for alkaloid supply.

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Keywords Antioxidant · *Catharanthus roseus* · Hydrogen peroxide · Monoterpene indole alkaloids · *Psychotria* · Reactive oxygen species

Introduction

Secondary metabolites, are widely present in the photosynthetic lineage of organisms, generally playing a major role in plant ecochemical interactions. Synthesis of secondary metabolites in plants is one of the main strategies to overcome the ever-changing environmental conditions such as light intensity and quality, temperature variation, soil conditions (i.e. water content, salinity, nutrient variation, metal presence, and microorganism interaction), competing neighbor plants, and pathogen and herbivore attacks. Secondary metabolites can also be regulated by the circadian clock and development, changing during both vegetative and reproductive growth (Nascimento and Fett-Neto 2010).

Production of secondary metabolites involves different pathways, several of which can be stimulated by oxidative stress. The impact of oxidative stress is not only evident under field conditions, triggered by biotic and abiotic signals, but also in controlled bioreactor environments. Aeration and agitation in bioreactors for plant cell and organ cultures, required for maintaining aerobic conditions and homogeneity of mass and heat transfer, as well as nutrient and metabolite distribution, can eventually increase oxidative stress

and influence both growth and secondary metabolism (Georgiev et al. 2009). Interestingly, low levels of oxygen (anoxic shock) may also increase oxidative stress in cell cultures (Nisi et al. 2010).

After specific environmental conditions or under specific stress factors exposure, phytoalexin-like compounds may be elicited to accumulate. In contrast, phytoanticipin-like compounds are present constitutively. Many metabolites present an accumulation profile somewhat in between these extremes. These elicited or constitutive compounds act directly (bitterness, toxicity, repellent properties, proteinase inhibitors) or indirectly (intra- and inter-specific signaling and internal signaling, antioxidant compounds), contributing to overall plant resistance. Antioxidants play a fundamental role alleviating or spatially restricting oxidative stress generated upon challenging conditions (Matsuura and Fett-Neto 2013). A significant fraction of plant antioxidants includes secondary metabolites, which may play a key role in plant adaptation to biotic and abiotic stresses, as a protective mechanism against oxygen species which can damage membranes, organelles and macromolecules.

The role of natural products in the adaptation of the plant to the environment and the need for in situ responses of plants due to the sessile condition, drives the great chemical variety and complexity of these molecules. These then represent a major reservoir of potential new drugs (Matsuura et al. 2013). Among the classes of bioactive secondary metabolites, alkaloids are of great significance.

Alkaloids

Alkaloids are N-containing molecules of diverse biosynthetic origin found in ~20 % of plant families. Various alkaloids with elucidated structures exhibit biological activity, such as antitumoral, analgesic, antimicrobial and insecticide. The main ecological role proposed for alkaloids due their toxic properties is protection against herbivores and pathogens (Roepke et al. 2010). Evolution of compounds towards specific functions (e.g. protection against predators) often confers additional properties that improve plant fitness to various extents, such as antioxidant activity. Several studies report antioxidant activity for alkaloids, which may not be directly linked to defense against herbivores but may participate in overall plant antioxidant processes to cope with oxidative stress.

Monoterpene indole alkaloids (MIAs) are alkaloids originating from the condensation of tryptophan (shikimate pathway) and a terpene moiety (often derived from the methylerythritol phosphate pathway, but also from the mevalonate pathway) by the action of strictosidine synthase (STR) (Simkin et al. 2013) or some STR-like enzyme. MIAs are found in Apocynaceae, Loganiaceae, Rubiaceae and Nyssaceae. *Catharanthus roseus* (Apocynaceae) is considered a key species for the study of MIAs metabolism. Indole alkaloids include important bioactive compounds, such as ajmalicine from *Rauwolfia serpentina* (cardiopathy treatment), camptothecin from *Camptotheca acuminata*, and vincristine and vinblastine from *C. roseus* (antitumoral agents).

Vinblastine and vincristine contents in *C. roseus* range from 0.0003 to 0.01 % dry weight (DW), depending on growth conditions (Liscombe and O'Connor 2011). The pharmacological importance of MIAs is the main motivation for research on the control of their biosynthesis. Sustained supply of bioactive MIAs faces hurdles, such as limited availability in plant tissues and the complex structures that often make chemical syntheses not a commercially viable option.

Understanding alkaloid metabolism enables the manipulation of key steps to obtain higher levels of alkaloids. However, biosynthesis of MIAs is highly complex and involves various enzymatic steps occurring in different cell compartments and with portions of a pathway being expressed in different tissues and cell types (Nascimento and Fett-Neto 2010). The intra and intercellular partitioning of MIA biosynthetic pathways requires the action of metabolite transporters and adequate protein targeting, both operating in accordance with appropriate substrate availability (Heinig et al. 2013). Biotechnological research aiming at higher yields for plant compounds exhibiting interesting pharmacological activity remains a major focus for the pharmaceutical industry.

Experimental systems

Cell cultures often allow tightly controlled genetic and physiological manipulations, fast growth and alkaloid production independent of outdoor environmental

conditions. However, lack of cell differentiation can prevent alkaloid accumulation. Low irradiance and exogenous sugar availability may impair autotrophic metabolism-related genes and lead to poor development of functional chloroplasts, essential for some steps of indole alkaloid biosynthesis, resulting in low yields even in differentiated or whole plant in vitro cultures. Fast growing cells may also lack the capacity of producing alkaloids due to a number of possible factors, such as competition between primary and secondary metabolism, feedback inhibition due to small vacuoles and inadequate storage compartments, faulty expression of key regulatory transcription factors, selective proteolytic machinery or biosynthetic enzymes (Pasquali et al. 2006; Glenn et al. 2013). In *Psychotria*, callus and cell cultures were not capable of accumulating MIAs and, at least for *Psychotria umbellata*, the ability to synthesize alkaloids was closely associated with shoot differentiation (Paranhos et al. 2005).

Hairy and non-transformed root cultures are also frequently used to study MIA metabolism. These organ cultures share some features with cell cultures, including the relatively rapid growth, ease of manipulation, and amenability to cultivation in bioreactors (Georgiev et al. 2012). Hairy roots are also genetically stable and able to grow in the absence of exogenous phytohormones (Hussain et al. 2012). Direct exposure to oxidative stress (addition of H_2O_2 at 200 mM) on cell suspensions and root cultures of *Uncaria tomentosa* grown in bioreactors increased the indole alkaloid content (Huerta-Heredia et al. 2009). Forced aeration (dissolved O_2 excess) increased ajmalicine content in *C. roseus* cultures (Mujib et al. 2012), which could be linked to ROS formation. Oxidative stress can affect monoterpene alkaloid production and also can increase activities of key enzymes in MIAs biosynthesis such as STR and strictosidine glucosidase (SGD) in root cultures of *U. tomentosa* (Vera-Reyes et al. 2013).

Strategies for MIA production in whole plant systems to enhance alkaloid content include elicitation by UV exposure (pre or post-harvest), salicylic and jasmonic acid (JA) treatment, mechanical wounding, drought, salinity, temperature changes, light intensity and nutrient source variation (Nascimento and Fett-Neto 2010). Experimentation in whole plant system is a useful tool to understand the mechanisms of alkaloid biosynthesis *in planta*, particularly for metabolites that fail to be produced in heterotrophic or undifferentiated cultures (Pasquali et al. 2006).

Genetic transformation of plant cells, organs, or whole plants is useful for improving MIA biosynthesis and accumulation efficiency. Overexpression of key genes in MIA pathway [tryptophan decarboxylase (TDC), STR] are normally not sufficient to increase final alkaloid yield; usually, only the direct downstream product has increased levels with the content of MIAs of interest remaining unaffected. Better success rates may be achieved by increasing the expression of transcription factors, such as members of the ORCA family, which regulate several genes encoding biosynthetic enzymes (Shoji and Hashimoto 2013). Manipulation of metabolite transporters may also prove useful (Heinig et al. 2013).

ROS dual role: triggers and components of signaling pathways

After the perception of biotic and abiotic cues by plant cells, signaling pathways are activated leading to a variety of possible events, including phosphorylation cascades, ion channel activity, accumulation of JA, salicylic acid (SA), abscisic acid (ABA), ethylene, nitric oxide (NO) and reactive oxygen species (ROS) generation; the most frequent outcome of such changes is the modified expression of genes involved in plant defense. Phytohormones play important roles in abiotic (e.g. ABA) and biotic (e.g. JA, SA, NO and ethylene) stress signaling. JA is of key importance in signalling pathways leading to alkaloid production (De Geyter et al. 2012). Second messengers such as Ca^{2+} and ROS also play important roles in plant responses to stimuli that may result in secondary metabolite production (Fraire-Velázquez et al. 2011).

In the early response of plants to various environmental conditions, Ca^{2+} and ROS levels rapidly increase in plant cells, activating calcium-interacting proteins [e.g. calmodulin, calcium-dependent protein kinases (CDPKs) and calcineurin B-like proteins (CBLs)]. Crosstalk between Ca^{2+} and ROS is frequent, eventually modulating processes such as localized growth and defense responses. Ca^{2+} influx into the cytoplasm is stimulated by ROS, activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, establishing a positive feedback loop (Takeda et al. 2008). The oxidase activity can

generate superoxide anions, which are rapidly converted to H_2O_2 by superoxide dismutase (SOD) activity (Ramani and Chelliah 2007). Hydrogen peroxide may diffuse between cells and subcellular compartments (Agati et al. 2012).

Besides their toxic effects, ROS play an important role as second messengers (due to disturbance of redox status of the cell). Plants are able to sense and translate ROS signals into appropriate cellular responses that may later alleviate stress effects. These responses require redox-sensitive proteins which undergo oxidation/reduction cycles, acting as switches that report cellular redox state, directly modulating corresponding metabolism in the case of a redox-sensitive enzyme or in a redox-sensitive protein, and/or driving downstream signaling via cascades of kinases, phosphatases and transcription factors (Raina et al. 2012). The main signaling cascades involve mitogen-activated protein kinases (MAPKs), protein kinases of the Ser/Thr family widely conserved among eukaryotes. These kinases respond to extracellular stimuli and regulate intracellular responses through amplification of the transduced signal. At the end of this series of events, modified levels of several metabolites and proteins can be detected. ROS interference in the expression of several genes and signal transduction pathways has been widely reported (Ahmad et al. 2010).

Oxidative stress

Oxidative stress is the condition in which the production of reactive species exceeds antioxidant defenses thereby disturbing the redox balance. In face of different environmental challenges, one of the primary effects is ion unbalance and localized hyperosmotic environments, leading to high energy state electrons that are transferred to molecular oxygen, resulting in reactive forms such as singlet oxygen, hydroxyl radicals, superoxide ions, and peroxides (e.g. H_2O_2). These are highly reactive chemical species that may cause damage to DNA and membranes, but also unleash a molecular network that activates responsive mechanisms to protect and repair damaged sites. Antioxidant defenses can be of enzymatic or chemical nature. Superoxide radical has short half-life and is rapidly converted to H_2O_2 by SOD. H_2O_2 is a relatively stable product that can be detoxified by catalase and peroxidases (PODs) (Jaleel et al. 2008). The non-enzymatic defense realm is where vitamin E, glutathione and an array of secondary metabolites can play roles to mitigate excessive ROS.

The use of O_2 in biological systems is a trouble/opportunity balance that contributed to shaping the evolution of life. Increased biological complexity and compartmentalization required a refined system of communication among cells, tissues and organs. Redox cues (antioxidants and reactive species of oxygen, nitrogen, carbonyl and sulfur) regulate protein synthesis, ROS-regulated transcription factors and thioredoxins redox regulation of photosynthesis (De Tullio 2010). Redox regulation is a sensitive and general mechanism able to perceive small changes in the environment, gating specific adaptive responses (De Tullio and Asard 2012).

Oxidative stress as a switch for MIAs production

Changes in redox balances trigger signaling cascades that may result in alkaloid production. There are a few reports directly linking oxidative stress and MIA biosynthesis. Cells of *C. roseus* exposed to osmotic stress showed changes in ROS-related metabolism (lipid peroxidation, H_2O_2 content and free-radical quenching systems, including both enzymatic and non-enzymatic antioxidants), leading to higher ajmalicine content (a serpentine precursor) in comparison to control cells (Zhou et al. 2009). Cell surface receptors, Ca^{2+} influx, medium alkalinization, H_2O_2, CDPK and MAPK were also shown to play significant roles in signaling under UV-B stress, resulting in higher TDC and STR gene transcription, and in the accumulation of catharanthine in cell suspension cultures of *C. roseus* (Ramani and Chelliah 2007).

The antitumoral alkaloid vinblastine is synthesized through vindoline and catharanthine coupling by α-3′,4′-anhydrovinblastine (AVBL) synthase, in an oxidation process leading to the unstable precursor of vinblastine, AVBL. Specific in vitro AVBL synthase activity was found for a H_2O_2-dependent POD present in vacuoles of mesophyll cells (Kumar et al. 2007), supporting a correlation between plant cell redox state and vinblastine biosynthesis.

Plant POD isoenzymes may participate in oxidation or biosynthesis of phenolics, hormones and alkaloids in presence of H_2O_2, playing important roles in plant

adaptation to environmental cues (Ferreres et al. 2011; Zipor and Oren-Shamir 2013). Exogenous addition of H_2O_2 can lead to rapid endogenous H_2O_2 production, disturbing redox state (Tang et al. 2009). Application of exogenous H_2O_2 at 100 mM in *C. roseus* seedlings promoted POD activity and approx. doubled the content of vindoline, catharanthine and vinblastine and quadrupled the AVBL content. Oxidative stress and alkaloid production were significantly correlated, which was particularly evident between POD activity and AVBL synthesis (Tang et al. 2009).

Experiments with different elicitors of acetyltransferase, POD, hydroxylase and inhibitors of oxygenase added to *C. roseus* cell culture medium to investigate their effects on the biosynthesis of tabersonine, vindoline and vinblastine found H_2O_2 as the most effective agent for enhancing tabersonine content. 7 days after the addition of H_2O_2 (20 μg l^{-1}) to the cell suspension culture, tabersonine content reached 9 mg g^{-1} DW (Guo et al. 2013). Tabersonine may later yield vindoline.

In *C. acuminata* cell cultures, H_2O_2 was also effective in enhancing about 400 % the content of 10-hydroxycamptothecin compared to control cultures, with a concomitant decrease in camptothecin content. UV-B radiation, a strong inducer of oxidative stress, caused an order of magnitude increase in camptothecin content of treated cells (Pi et al. 2010). Similar increase was reported for brachycerine in leaves of *Psychotria brachyceras* upon acute exposure to UV-C (Gregianini et al. 2003). Acute exposure of plants to UV-B doubled the brachycerine content, which was associated with early induction of alkaloid-biosynthesis related genes (Nascimento et al. 2013a). In *C. acuminata* seedlings, higher camptothecin content and number of glandular trichomes containing the alkaloid were observed upon drought stress (a typical ROS-inducing condition) (Valletta et al. 2010). Brachycerine accumulation in leaf disks of *P. brachyceras* was induced by exposure to osmotic stress agents, ABA and heavy metals (Nascimento et al. 2013b).

Alkaloids may act as an efficient substrate for POD, antagonizing oxidative environmental stress, indicating a link between MIA production (e.g. vinblastine and its precursors) and ROS burst (e.g. H_2O_2) induced by exogenous stress signals. Non-MIA alkaloids, such as nicotine, also have their concentration increased in cells and tissues upon oxidative stress induced by herbivory, for example, often mediated by JA (De Geyter et al. 2012).

Despite its overt potential for alkaloid elicitation, oxidative stress is harmful to biological systems and the contents of ROS in plant manipulation have to be strictly controlled. In *C. roseus*, the biosynthesis of catharanthine and serpentine is inhibited when the concentration of H_2O_2 is excessive, probably as a function of damage to cells and biosynthetic machinery by high ROS activity (Tang et al. 2009). These results highlight the importance of antioxidant molecules.

Antioxidants

Antioxidants play a key role to the redox balance in cells, be them of enzymatic or non enzymatic nature. Enzymatic antioxidant defenses include ascorbate peroxidase (APX), SOD, CAT, glutathione peroxidase, and glutathione reductase (GR), whereas non enzymatic antioxidant molecules include tocopherol, ascorbic acid, glutathione and secondary metabolites, including MIAs.

There is a lack of consensus whether antioxidants act individually or synergistically to regulate the redox environment, or both. Studies focusing on individual antioxidants show a non interchangeable nature among these agents. In some cases, however, an antioxidant can reinforce the action of another. The spotlight has been long on ascorbate and glutathione and, in recent decades, increasing attention has been given to tocopherols and carotenoids (Gill and Tuteja 2010). Little is known about the physiological contribution of antioxidant secondary metabolites other than flavonoids to the redox environment of plant cells (Agati et al. 2012). MIAs with antioxidant properties, upon elicitation or in basal concentration, may contribute to the overall cell redox state at least to a certain extent.

Some MIAs can be substrates for PODs, such as vindoline and catharanthine, generating AVBL and subsequently vinblastine. These dimeric alkaloids may antagonize oxidative stress, providing evidence of a direct link between alkaloid production and ROS burst and that MIAs may serve as antioxidants in general plant protection responses (Tang et al. 2009).

Significant antioxidant properties have been shown for brachycerine, psychollatine and *N*-β-D-glucopyranosyl vincosamide (GPV), all of them MIAs found in

Table 1 Representative examples of MIA for which antioxidant properties have been shown

Plant	Alkaloid	Antioxidant activity	Reference
C. alata	1-(4′-Hydroxyphenyl)-2,4,6-trihydroxy-indole-3-carboxylic acid	DPPH test	Olarte et al. (2010)
P. brachyceras	Brachycerine	Singlet oxygen, superoxide, hydroxyl radicals, hydrogen peroxide	Gregianini et al. (2003), Nascimento et al. (2007), Porto et al. (2009)
P. leiocarpa	GPV	Singlet oxygen, superoxide, hydroxyl radicals, hydrogen peroxide	Matsuura and Fett-Neto (2013)
P. umbellata	Psychollatine	Singlet oxygen, superoxide, hydroxyl radicals, hydrogen peroxide	Fragoso et al. (2008), Paranhos et al. (2009)
R. serpentina	Reserpine	Singlet oxygen	Larson and Marley (1984)
R. serpentina	Serpentine	Cell based assay (cell line F-HABP07)	Dutta et al. (2011)
Strychnos sp.	Brucine	Singlet oxygen	Larson and Marley (1984)
Strychnos sp.	Strychnine	Singlet oxygen	Larson and Marley (1984)
V. minor	Vincamine	Singlet oxygen	Larson and Marley (1984)
V. minor	Vinpocetin: semisynthetic derivative of vincamine	Hydroxyl radicals	Oláh et al. (1990)

subtropical *Psychotria* species of Brazil, against the main ROS found in plants (Table 1). An antioxidant role for MIA in plant protection was also proposed for GPV, the major MIA in *P. leiocarpa*, that was unable to prevent generalist herbivore feeding, showing instead a broad and strong antioxidant activity (H_2O_2, hydroxyl radical, superoxide anion and singlet oxygen scavenging properties); addition of GPV to a different species (heterologous system) under acute UV-B lead to improved plant fitness maintenance, reinforcing a general antioxidant protection role (Matsuura and Fett-Neto 2013). Other *Psychotria* alkaloids, such as brachycerine from *P. brachyceras*, displayed in vitro scavenging activity against H_2O_2, hydroxyl radical, superoxide anions and singlet oxygen (Gregianini et al. 2003; Nascimento et al. 2007; Porto et al. 2009). Psychollatine of *P. umbellata* was effective to protect against acute UV-C damage and acted as in vitro antioxidant against singlet oxygen, hydrogen peroxide, superoxide (Paranhos et al. 2009) and hydroxyl radical (Fragoso et al. 2008). In the case of acute UV-stress protection by MIAs, evidence of chemical quenching by brachycerine and psychollatine was provided through chromatographic analyses of catabolites (Gregianini et al. 2003, Paranhos et al. 2009).

Some of the alkaloids of *Psychotria* of southern Brazil (brachycerine, GPV, and psychollatine) have common features that are relatively infrequent in various other MIAs, including the maintenance of glucose residues and accumulation of relatively high alkaloid concentrations in shoots (highest contents ranging from 0.3 to 4 % of leaf DW) (Porto et al. 2009). These characteristics, coupled to the relevant antioxidant properties of these metabolites, their apparent lack of deterrence or toxicity, and the induction of accumulation by stimuli causing oxidative stress for those that have a phytoalexin-type of accumulation, suggest that antioxidant activity is a relevant function for these metabolites.

In brachycerine, singlet oxygen quenching activity was quantified and lower but comparable to that of DABCO (1,4-diazabicyclo[2.2.2]octane), one of the fastest aliphatic–alicyclic tertiary amine quenchers known. Alkaloid structures contain several chemical groups potentially capable of quenching singlet oxygen, such as the nitrogen atoms of the indole ring and conjugated with the indole nucleus, double bonds, OH-group and the glucose residue (Fig. 1) (Gregianini et al. 2003). Similar chemical characteristics are found in psychollatine and GPV. Besides the efficient in vitro antioxidant activity against various ROS, both

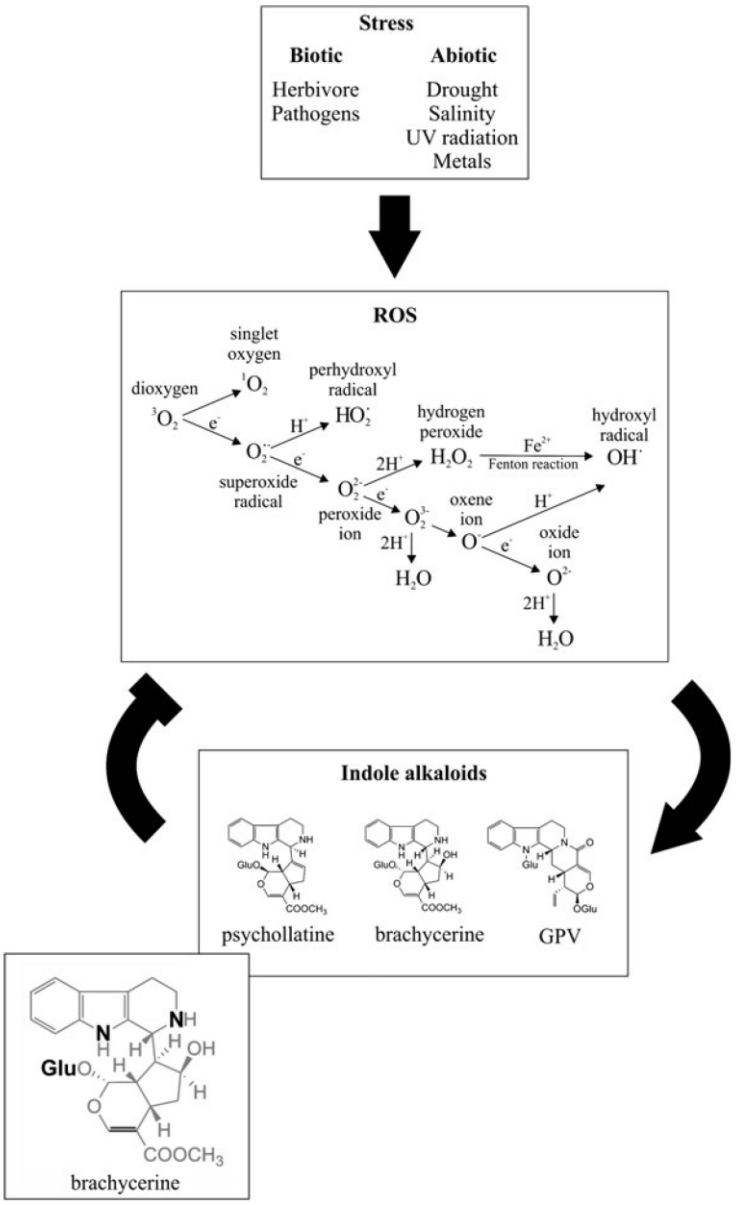

Fig. 1 Potential interactions involving environmental stresses, ROS generation, production of MIAs, and antioxidant defense response. Biotic and abiotic challenges activate ROS chemistry in plant cells, yielding a transient oxidative imbalance. ROS, particularly hydrogen peroxide, stimulate alkaloid biosynthesis and accumulation. Alkaloids, herein exemplified by MIAs of some Neotropical *Psychotria* (psychollatine, brachycerine and GPV), may act as part of the overall antioxidant defenses of the plant to re-establish adequate oxidative balance. Some of the chemical features that allow MIAs to play this role, for example against singlet oxygen, are distinguishable in the molecule of brachycerine (see text for details)

brachycerine and psychollatine have shown in vivo antioxidant activity against H_2O_2, being capable of protecting yeast strains deficient in the enzymatic antioxidant system against the mutagenic action of this ROS (Nascimento et al. 2007; Fragoso et al. 2008).

Antioxidant activity was shown for serpentine in a cell-based assay using a stable line of murine fibroblasts overexpressing human hyaluronan binding protein1 (HABP1), which accumulates in mitochondria leading to excessive ROS generation. Serpentine was characterized as a non-cytotoxic antioxidant (Dutta et al. 2011). The indole alkaloid, 1-(4′-hydroxyphenyl)-2,4,6-trihydroxy-indole-3-carboxylic acid, from *Cassia alata* had a dose-dependent scavenging activity against diphenyl picryl hydrazyl hydrochloride (DPPH), displaying strong antioxidant potential (IC_{50} of 0.031 μM ± 0.002) (Olarte et al. 2010). Representative examples of MIAs for which antioxidant properties have been shown are listed in Table 1.

A careful investigation on the subcellular localization of STR and strictosidine β-D-glucosidase (SGD) in *C. roseus* revealed a spatial separation of these enzymes in the vacuole and the nucleus, respectively, as well as an accumulation of strictosidine in the former compartment (Guirimand et al. 2010). MIA production would be limited by the transport of strictosidine to the nucleus, where SGD accumulates as stable supramolecular aggregates. Only after the action of SGD, is the aglycone formed which then allows alkaloid biosynthesis to proceed. Upon damage to cells and membrane compartments, increased co-localization of strictosidine and SGD would lead to formation of the aglycone and its derivative, the reactive dialdehyde, causing massive protein cross-linking and precipitation by the non-metabolized dialdehyde, presumably affecting the function of herbivore or necrotrophic pathogen enzymes (Guirimand et al. 2010). It is not clear, however, if part of the activated dialdehyde pool could also support increased alkaloid production in local or adjacent tissues, as part of the defense response. Participation of various subcellular compartments in MIA biosynthesis and pathway distribution through various cell types and tissues with transport of metabolic intermediates have important implications for their feasible role as potential antioxidants in oxidative stress responses, as discussed for flavonoids (Agati et al. 2012).

Vincamine of *Vinca minor*, used as an anti age-related disease agent, caused a 50 % decrease in iron content of rat brain, possibly decreasing oxidative stress caused by accumulation of this metal during neurodegenerative processes (Fayed 2010). Additionally, the semi-synthetic MIA vimpocetine (produced from vincamine) inhibited dopamine-quinone products, free radical formation and lipid peroxidation in brain striatum synaptosomes (Herrera-Mundo and Sitges 2013). Although the concentrations of individual MIAs in some plants, such as *C. roseus*, are often very low, the sum of all 130 alkaloids or so produced and accumulated by this species (Ferreres et al. 2011) may have a significant impact on its redox state.

The indole ring in MIAs may be relevant to explain antioxidant activity of the molecule. A high correlation with scavenging activity of peroxyl radical by indole compounds was found in a study of the electrochemical behavior of compounds from an indole library, including tryptophan and tryptamine derivatives, with previously known activity against ROS. Voltammetry and oxidation potential of the molecule correlated with scavenging activity (Estevão et al. 2011). Screening and mechanism-based investigation of antioxidant activity of alkaloids could have implications both for the better understanding of plant protection mechanisms against stresses and for unveiling additional pharmacological uses for MIAs.

Conclusions

Due to the relevant pharmacological properties of some MIAs, their relatively low natural yields in plants, and structural complexity that prevents economically viable chemical synthesis, detailed understanding of factors affecting alkaloid biosynthesis is needed. Oxidative stress is a common outcome of biotic and abiotic stresses, and may lead to several responses including antioxidant systems activation and secondary metabolites elicitation, processes that may be interconnected for successful protective responses.

An array of antioxidant compounds (e.g. ascorbic acid, tocopherols, glutathione, phenolics and alkaloids) contributes to various extents in the overall redox state, coupled to the enzymatic antioxidant system, playing a negative feedback role on ROS toxic effects, thereby improving plant fitness under stress. H_2O_2 is the main stable ROS, being rapidly formed after stress and capable of acting directly or indirectly as a second messenger, alone or in crosstalk with Ca^{2+}, affecting signaling cascades and resulting in physiological responses, including MIAs biosynthesis, which would be critical in increasing plant protection. Information on MIAs biochemistry provides, at least in part, some critical information for this class of secondary metabolites, including: (1) overall antioxidant properties, (2) cellular concentration of major or total types, (3) distribution through various subcellular compartments of intermediates and/or final products, and (4) the apparent involvement in oxidative stress responses. Taken together, the evidence indicates that MIAs can play a role in antioxidant responses of plants facing stresses.

Regulating MIAs biosynthesis by applying mild to moderate oxidative stress makes use of a general plant response to biotic and abiotic challenges, providing a relatively low cost and easy to apply biotechnological strategy to obtain higher yields of bioactive alkaloids of interest.

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Isolation and characterization of cyclotides from Brazilian *Psychotria*: significance in plant defense and co-ocurrence with antioxidant alkaloids.

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Isolation and characterization of cyclotides from Brazilian *Psychotria*: significance in plant defense and co-occurrence with antioxidant alkaloids

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Abstract

Psyleio A is the major cyclotide present in leaves of *Psychotria leiocarpa*. This peptide, also found in *Psychotria brachyceras* leaves in lower concentrations, showed insecticidal effects on *Helicoverpa armigera* larvae. Both plant species had a rich pool of cyclotides, indicating a possible role of these peptides in protection against herbivores. High contents of antioxidant indole alkaloids, previously shown as not being involved in herbivore deterrence or pathogen inhibition, co-occurred with the cyclotides. The contents of major cyclotides and alkaloids from both *Psychotria* species were monitored after herbivore and pathogen-related treatments, revealing a constitutive, phytoanticipin-like accumulation pattern for all cyclotides analyzed in each species. The control treatment with ethanol used as vehicle in larvae trials for solubilizing alkaloids was somewhat toxic. Improved larval weight gain and survival was observed in treatments containing alkaloids. This suggests that ethanol-induced oxidative stress was mitigated by alkaloids, corroborating, in a heterologous system with artificial oxidative stress stimulation, the antioxidant efficiency of *Psychotria* alkaloids as observed *in planta*. This study reports data on eight novel cyclotides, identification of *Psychotria leiocarpa* as a cyclotide bearing species, and absence of these peptides in *Psychotria umbellata*.

Keywords. cyclotide, insecticidal, monoterpene indole alkaloid (MIA), antioxidant, psyleio, psybry, *P. leiocarpa*, *P. umbellata*

Introduction

Rubiaceae is among plant families producing both cyclotides and alkaloids, with important representatives of each class of compounds such as *Oldenlandia affinis* (1) and *Coffea arabica* (2), respectively. *Psychotria* is the largest genus among Rubiaceae, being globally distributed in the tropics and subtropics, having a complex phylogeny (3, 4, 5).

Cyclotides represent the major group of circular proteins naturally occurring in plants (6, 7). The main ecological role credited for cyclotides relates to their insecticidal properties (8, 9), presumably acting by rupture of midgut epithelium cells (10) and interaction with membrane lipids in a receptor-independent manner (11), a feature that may prevent insects from acquiring resistance (12, 13).

Cyclotides are highly stable small circular peptides. Containing 17 to 39 amino acids (www.cybase.org.au), cyclotides are characterized by 6 cysteines of 3 disulfide bonds organized in a "cysteine knot" configuration (14), often having a conserved glutamic acid (Figure 1) (15). These peptides may be potential scaffolds in the design of target-specific drugs (12, 16). Biological activities described for these compounds include uterotonic, anti-HIV, antimicrobial, insecticidal and molluscicidal activities (10, 17, 18, 19, 20).

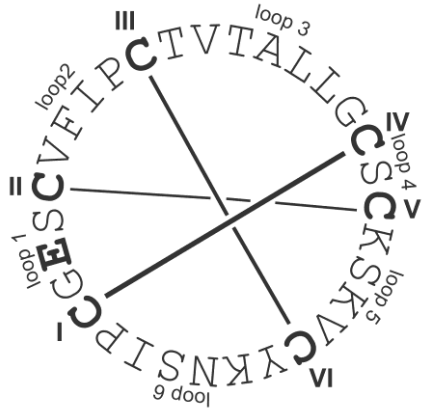

Figure 1. Cyclopsychotride A from *Psychotria vellosiana* (formerly *P. longipes*), the first cyclotide described in the genus *Psychotria*

To date, 16 cyclotide sequences are known from eight *Psychotria* species, *P. vellosiana* (cyclopsychotride A) (15), *P. suterella* (PS-1) (6), *P. leptothyrsa* (psyles A-F) (21), *P. brachiata* (psybra 1), *P. deflexa* (psydef 1 and 2), *P. poeppigiana* (psypoe 1), *P. suerensis* (psysue 1 and 2), and *P. solitudinum* (psysol 1 and 2) (22, 23). Six cyclotides (caripe 1-6) are described from *Carapichea ipecacuanha*, formerly *Psychotria ipecacuanha* (22). Other

species with undescribed cyclotides are *P. brachyceras*, *P. buchtienii*, *P. chiriquiensis*, *P. elata*, *P. goldmanii*, *P. mortoniana*, *P. pilosa*, *P. prunifolia*, *P. punctata* and *P. trichophora* (6, 22). Sixty-one *Psychotria* species apparently lack cyclotides (6, 21, 22). Bioactivities found in *Psychotria* cyclotides include cytotoxic activity to MCF-7 and MCF-7/ADR cell lines for psyle A, C and E (*P. leptothyrsa*) (24), antimicrobial properties and hemolytic activity against human red blood cells for cyclopsychotride A (*P. vellosiana*) (17), and inhibitory activity against prolyl-oligopeptidase by psysol 2 (*P. solitudinum*) (23).

Psychotria spp. are rich sources of alkaloids (25). Alkaloids are often anti-herbivore defenses, but these metabolites may also be relevant players in oxidative stress detoxification and control rather than herbivore deterrence (26). As defense molecules, alkaloids can be neurotoxic or disrupt cell signaling (27, 28). Shoots of southern Brazilian *Psychotria* accumulate significant concentrations of indole alkaloids (0.2% to 4.5% DW) with strong antioxidant activity, but these metabolites are not herbivore deterrents (29, 30, 31, 32, 33), a function which could be played by co-occurring cyclotides. This could result in a combined defense strategy, with antioxidant alkaloids, both inducible (34) and constitutive, acting in plant oxidative stress detoxification (29, 30) and cyclotides fulfilling the role of herbivore inhibition.

The present work focused on isolation, identification, and characterization of new cyclotides from *Psychotria*, as well as on their potential role in plant defense against insects. In addition, we also sought to better understand how cyclotide and indole monoterpene alkaloid productions are integrated in the plant defense framework of two of the most representative *Psychotria* species in the understorey of the southern Atlantic Forest (35), *P. leiocarpa* and *P. brachyceras*.

Experimental Procedures

Plant Material

Psychotria spp. were collected in Morro Santana- Federal University of Rio Grande do Sul (UFRGS) in the city of Porto Alegre, RS, Brazil, under harvest authorization Sisbio/ICMBio 32855-1 (authentication code: 58482685). Leaves of *P. brachyceras* Müll Arg., *P. carthagenensis* Jacq., *P. leiocarpa* Cham. & Schltdl. and *P. umbellata* Vell. were immediately frozen in liquid nitrogen after harvesting, freeze dried, and stored at -80° C until processing. Voucher specimens (7899- *P. brachyceras*; 7901- *P. carthagenensis*; 138157- P. *leiocarpa*; and 98869- *P. umbellata*) are deposited at the ICN herbarium (UFRGS). Freeze dried material was pulverized for analysis and isolation steps (referred to as dried plant

material). For wounding, jasmonate and salicylate exposure assays, shoots of *P. leiocarpa* and *P. brachyceras* containing 6 to 8 leaves were harvested and acclimated for 5 days under controlled conditions [10% Murashige & Skoog nutrient solution (36) pH 5.8, 16 h per day of photoperiod at 73 µmol m^{-2} s^{-1} of photosynthetically active radiation and 25 ± 3° C] prior to treatment application.

Extraction

Extraction of cyclotides from *P. leiocarpa* and *P. brachyceras* was performed based on the protocol described by Mahatmanto et al. (2014) (37) with minor modifications. A solution of acetonitrile: water: formic acid (25:24:1) [acetonitrile (Millipore, Billerica, MA); formic acid (Sigma-Aldrich, St. Louis, MO)] was added to dried plant material (1:20; m/v) and extract by 1-hour with agitation in magnetic stirrer. The extract was filtered through qualitative filter paper, centrifuged at 5500xg for 10 min at room temperature and the supernatant washed with dichloromethane (1:1; v/v); the aqueous layer had most of organic solvent removed by rotatory evaporation (below 45° C), and then it was filtered through 0.45 µm membrane before freeze drying (product referred to as crude extract).

Reduction, Alkylation and Enzymatic Digestion of Extracts

Plant extract was obtained by dissolving dried plant material (*P. brachyceras*, *P. carthagenensis*, *P. leiocarpa* and *P. umbellata*) in 20% acetonitrile (Sigma-Aldrich, St. Louis, MO) and 1% formic acid (Sigma-Aldrich, St. Louis, MO) solution. Reduction of disulfide bonds was performed by adding 100 mM ammonium bicarbonate buffer (Sigma-Aldrich, St. Louis, MO) (1:1; v/v) (pH 8.0), and 100 mM dithiothreitol (DTT) (Astral, Gymea, NSW) to samples (1:10; v/v) before incubation at 60° C for 30 min; alkylation of reduced cysteine residues were performed by addition of 250 mM iodoacetamide (Sigma-Aldrich, St. Louis, MO) to reduced samples (1:10; v/v) and incubation for 20 min at room temperature. Reduced and alkylated samples were enzymatically digested by adding 50 ng μL^{-1} of either trypsin (Sigma-Aldrich, St. Louis, MO) or endoproteinase GluC (Sigma-Aldrich, St. Louis, MO) or both to samples (1:1; v/v) and incubating for 3h at 37° C; the reaction was quenched by adding 1% formic acid (1:4; v/v) to samples. All the samples were desalted using C18 Zip Tips (Millipore, Billerica, MA) before MS analysis.

MALDI-TOF/TOF Analysis and Cyclotide Sequencing

MALDI-TOF/TOF analysis was performed in crude native, reduced and alkylated, and digested samples in a MALDI-TOF-TOF 4700 (AB Sciex, Framingham, MA) in positive reflector mode acquiring 1600-10000 total shots per spectrum with a 3700 laser intensity. α-cyano-hydroxy cinnamic acid (CHCA) (Protea, St. Louis, MO) 5 mg mL^{-1} in 50% (v/v) acetonitrile was used as matrix; 0.7 µL of matrix was added to 0.7 µL of samples, and 0.7 µL of the mixture was spotted on the MALDI plate. Spectra were acquired and processed by 4700 Analyzer Software, and manual sequencing was used to identify cyclotides. Additionally, sequencing by nanospray MS-MS in a QStar mass spectrometer was performed when needed. Spectra were acquired between m/z 60–2000 under a capillary voltage of 900 V for both TOF and product ion spectra; the collision energy for peptide fragmentation varied between 10 and 50 V, and the Analyst software program was used to acquire and process data (6). Peptides were sequenced based on the N-terminal b-ion and C-terminal y-ion fragmentation from the MS/MS spectra.

Cyclotide Purification

The crude extract from *P. leiocarpa* and *P. brachyceras* were solubilized in 10% acetonitrile (Sigma-Aldrich, St. Louis, MO) and processed in solid-phase extraction C18 columns (Agilent, St. Clara, CA). Sample elution from the column was done with solvent gradient by increasing the concentration of acetonitrile, starting with 20% acetonitrile 1% formic acid and finishing with 80% acetonitrile 1% formic acid (100 mL of each solution, containing 1% formic acid and acetonitrile 20, 30, 40, 50, 60, 70 or 80%). Fractions containing masses in cyclotide compatible range (checked by LC-MS) were combined (30% to 60% acetonitrile), and freeze dried. Combined dried fractions were resuspended in 10% acetonitrile and submitted to HPLC using preparative C_{18} column (Jupiter; 250 x 21.2 mm, 300 A°, Phenomenex) with a linear gradient starting with solvent A (0.05% TFA) and ending with solvent B (90% acetonitrile, 0.05% TFA), at 1% min^{-1} solvent B gradient and a flow rate of 8 mL min^{-1}. Peaks containing cyclotide masses (checked by LC-MS) were freeze dried, resuspended in 10% acetonitrile and submitted to semi-preparative HPLC (Jupiter C_{18} column; 250 x 10 mm, 300 A°, Phenomenex) in a linear gradient starting with solvent A and ending with solvent B, at 0.33% min^{-1} solvent B gradient and a flow rate of 3 mL min^{-1}.

Insecticidal assay

Helicoverpa armigera eggs were hatched and larvae grown under controlled diet until reaching between 4 to 11 mg. Before feeding trials, larvae were starved for 22 h (4 larvae per

treatment). The feeding trial was conducted for 72 h with larvae maintained at 25° C throughout the experiment, and the weights recorded at 0, 24, 48, and 72 h. Diets containing wheat germ, yeast, soy flour, agar and a set of anti-bacterials and anti-fungals (38, 39) were provided. Test diets contained the cyclotides psyleio A, psyleio B, or psybry A, or the alkaloids GPV, brachycerine, or psychollatine, at 1 μmol g^{-1} or 5 nmol g^{-1}; *P. leiocarpa*, *P. brachyceras*, *P. umbellata* and *P. carthagenenis* leaf extracts were also tested at 1.25 mg g^{-1}. Control diet did not have any added peptide or alkaloid. Psyleio B, the alkaloids, and the plant extracts were solubilized in 2% undenaturated ethanol due to solubilization issues in water only; a 2% undenaturated ethanol control without any peptides or alkaloids was also evaluated in this case.

Wounding assay

Previously acclimated shoots of *P. leiocarpa* and *P. brachyceras* had half of total leaves in shoots damaged 4 to 6 times by tweezers. Control plants remained intact in the same experimental conditions (same as acclimation treatment). Plants remained in the same room for 96 h; preliminary tests were made to check any possible secondary metabolism alteration due to stressed plant proximity and communication via volatile compounds, and no changes were observed. After 96 h, samples were harvest and immediately frozen in liquid nitrogen and stored at -80° C until processing.

Jasmonate and Salicylate assay

Leaf disks of 1cm diameter were prepared from previously acclimated shoots of *P. leiocarpa* and *P. brachyceras*. Thirty disks were distributed per Petri dish containing Murashige & Skoog solution at 10% strength, pH 5.8, in the absence (control) or presence of jasmonate or salicylate at final concentration of 400 μM. After 96 h of exposure, the samples were harvested, immediately frozen in liquid nitrogen and stored at -80° C until processing. The wounding inflicted in the procedure of making the leaf disks does not affect general metabolism of *P. leiocarpa* and *P. brachyceras*, as previously reported (29, 30).

Method of Standard Addition for indole alkaloids and cyclotide absolute quantitation

Levels of the main alkaloid and most abundant cyclotides from *P. brachyceras* and *P. leiocarpa* were evaluated in control and treated samples using the method of standard addition (40), allowing an accurate quantitation of each analyzed compound. Samples from wounding,

jasmonate and salicylate assay were dissolved in 50% acetonitrile 1% formic acid at a final concentration of 125 μg mL^{-1}, and injected onto a Shimadzu CTO 20A HPLC system (Shimadzu, Kyoto, Japan) with a flow rate of 4 μL min^{-1} in a Kinetex column (1.7μ C18 100A°, 100 x 2.10 mm) (Phenomenex, Torrence, CA). HPLC eluent was coupled directly to a 4000 Q-TRAP LC/MS/MS System (Applied Biosciences/MDS SCIEX, Foster City, USA) with a nano-electrospray ionization source were 5 specific standard compounds for each species monitored in samples by checking specific mass, and related main fragments, CE and DP, as follows: cyclotides psyleio A-D and the alkaloid GPV from *P. leiocarpa* samples, and cyclotides psybry A, psyleio A, B, D, and the alkaloid brachycerine from *P. brachyceras*. Source conditions were essentially set as previously described (41) with minor modifications. Standard compounds were used in final concentrations of 125, 250, 375 and 500 ng mL^{-1} and a non-related peptide, conotoxin, at final concentration 1000 ng mL^{-1} was used as internal standard. Data were acquired and processed using Analyst QS 2.02 software.

Experimental procedures and statistics

All assays herein described were performed in biological quadruplicates, and a technical duplicate whenever possible. The results were analyzed by Student´s t-test, or ANOVA followed by Tukey, $p < 0.05$, using GraphPad Prism 6 software.

Results and Discussion

Novel Cyclotides and Novel Cyclotide-Producing Plant

A new cyclotide containing, *P. leiocarpa*, and a new non-cyclotide producing species (*P. umbellata*), both from neotropical *Heteropsychotria* subgenus were found. We also report eight novel cyclotide sequences. So far, cyclotides have not been found in pantropical *Psychotria* species; the only exception is *P. punctata*, thought to be a cyclotide-containing species, but for which no sequence has been reported (22).

Of the four species analyzed in this study (*P. carthagenensis*, *P. brachyceras*, *P. leiocarpa* and *P. umbellata*), *P. leiocarpa* was found to be a novel cyclotide-producing plant, yielding at least eight cyclotides (Table 1). Five complete sequences were obtained through sequencing based on MS-MS data from MALDI and nanospray, all presenting novel structures. Evidence for cyclotides could not be found in *P. umbellata* leaves, thus including this as a new non-cyclotide producing species.

Table 1. Evidence for cyclotides in *Psychotria* species

Table 1. Evidence for cyclotides in *Psychotria* species

#	Peptide	Native mass (Da)	RA mass (Da)	Digested mass (Da)
		P. leiocarpa		
1	psyleio A	2988.06	3337.16	3355.15
2	psyleio B	2948.06	3297.16	3315.15
3	psyleio C	2921.04	3270.16	3288.12
4	psyleio D	2925.02	3274.12	3292.11
5	psyleio E	3026.03	3374.15	3392.14
6		3089.97	3437.09	-
7		3267.13	3615.24	3633.23
8		3289.10	3637.24	-
		P. brachyceras		
1	psyleio A	2987.85	3335.94	3353.91
2	psyleio B	2947.85	3295.92	3314.92
3	psyleio D	2924.80	3272.89	3291.87
4	psybry A	3289.03	3637.10	3655.07
5	psybry B	3271.01	3620.66	-
6	psybry C	3188.01	3536.09	-
7		2941.82	3289.90	-
8		3154.04	3502.12	-
9		3228.99	3576.03	3594.98
10		3232.99	3581.08	-
11		3282.14	3630.18	-
12		3292.02	3640.09	3658.09
13		3300.02	3648.12	-
14		3304.03	3653.10	-
15		3308.03	3656.10	-
16		3321.99	3670.07	-
17		3346.04	3694.13	-

P. brachyceras and *P. carthagenensis* were previously described as producing and not producing cyclotides, respectively (6, 21). In agreement with these data, cyclotides were present in *P. brachyceras* but not in *P. carthagenensis* evaluated in the present study. In a detailed screen of a cyclotide-rich extract, evidence for at least 17 cyclotides was obtained for *P. brachyceras*. Six complete sequences, being three novel and unique to *P. brachyceras*, three shared with *P. leiocarpa*, and one known sequence (cycloviolacin O17 from *Viola odorata*) were obtained (Table 1). Evidence of all *Psychotria* cyclotide sequences can be found in the supplemental material (Figures S1-S6).

New cyclotides were named following the nomenclature proposed by Broussalis et al. (2001) (42), using the first letters of the genus and the specific epithet of each species, and listed alphabetically. Cyclotides were ordered based on yields observed in plant material.

P. carthagenesis from southern Brazil lacks cyclotides, as reported for specimens of

different locations (6, 21), and is also devoid of alkaloids (43). However, some variations in metabolic profiles are described for plants originated from other regions. Specimens found in northern South America are used as a replacement of *P. viridis* as source of the psychoactive alkaloid N-N-dimethyltryptamine.

Cyclotide yields in plant leaves

Based on yields of the isolation protocol, *P. leiocarpa* had four major cyclotides, psyleio A-D. Psyleio A was present in a concentration of approximately 0.1% DW, and at least twice more abundant than psyleio B. *P. brachyceras* showed three most abundant cyclotides, psybry A, psyleio A and psybry B, being psybry B around 10-times less concentrated than psybry A (approximately 0.1% DW); psyleio B and D were found in *P. brachyceras* leaves in much lower concentrations. In Figure 2, the sequence of the first sequenced and isolated cylotide in this work, psyleio A, is shown, along with supporting evidence.

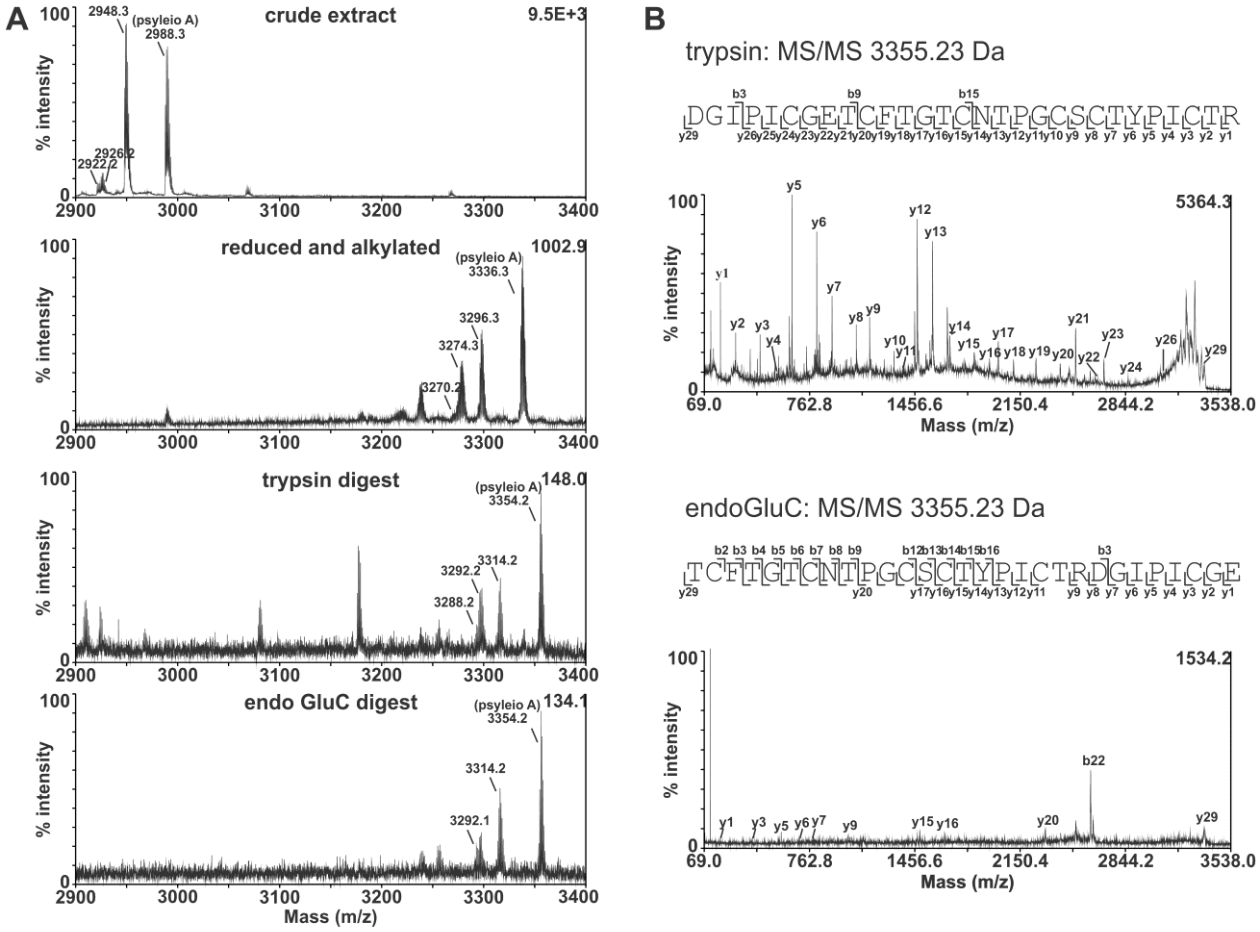

Figure 2. Characterization of the novel cyclotide psyleio A from *Psychotria leiocarpa*. (A) MALDI-MS of *P. leiocarpa* extract. The putative cyclotide mass of 2988.3 Da was selected as precursor for MS/MS, and reduced/ alkylated, trypsin digested, and endoproteinase GluC

digested. (B) MALDI-TOF/TOF sequencing of the precursor masses 3355.23 Da from a trypsin digest and an endoproteinase GluC allowed the characterization of the novel cyclotide psyleio A based on observed b- and y-ions. All shown masses are monoisotopic. MS evidence for other cyclotides in *P. leiocarpa* and *P. brachyceras* can be found in the Supporting Information section (Figures S1–S6).

Insecticidal activity

Psyleio A showed insecticidal properties against *H. armigera* larvae. For water based treatments, deaths (50%) were only observed in the presence of the cyclotide psyleio A (after 48 h, at concentration 1 μmol g^{-1}); the presence of the peptide negatively affected growth rate when compared to control larvae ($p < 0.01$) (Figure 3). Comparing the kinetics of larvae growth, except for psyleio A at 1 μmol g^{-1} treatment, in all other treatments larvae continuously gained weight showing the highest growth rate between 48 h and 72 h (Figure 3). Data indicated that psyleio A has insecticidal activity, and a 5 and 8 amino acid difference is observed in comparison to kalata B2 and kalata B1, respectively (*O. affinis*) (Table 2). A similar difference is observed comparing kalata B1 with kalata B2 (44) and both show insecticidal activity. Kalata B1 mode of action was demonstrated to be unique in the way it enters cells (11).

The most abundant cyclotide in *P. brachyceras* (psybry A, approximately 0.1% DW in leaves), did not affect growth or survival of *H. armigera*; yet, the presence of psyleio A among cyclotides shared between *P. brachyceras* and *P. leiocaropa* species could confer protection against insects. Moreover, psybry A could target different guilds of herbivores. Also, the pool of cyclotides reported in both plants (at least 6 untested cyclotides in *P. leiocarpa* and 14 in *P. brachyceras*) could enhance insecticidal activity in combined fashion.

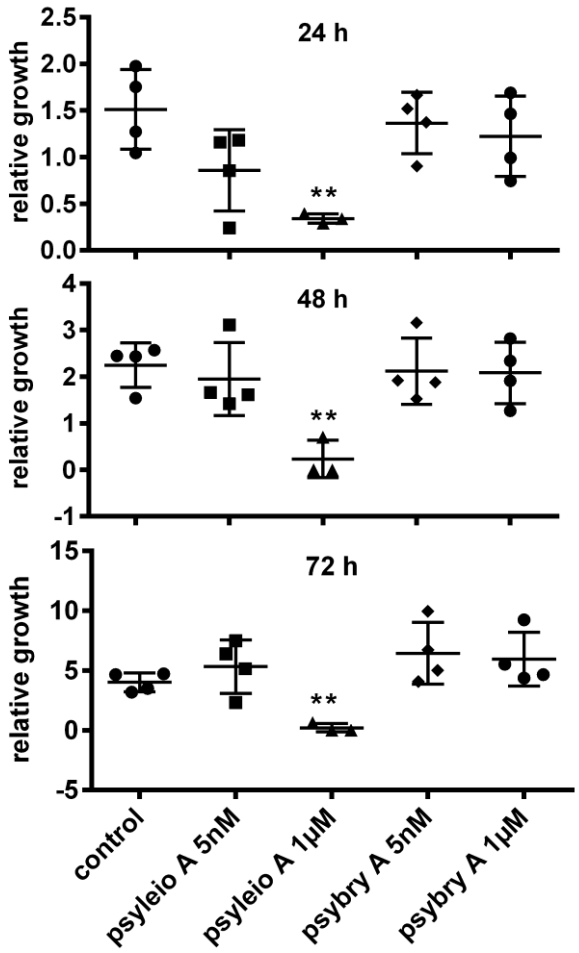

Figure 3. Insecticidal assay. The weight at 24, 48, and 72 h was compared to initial weight. Relative growth after 24, 48, and 72 h was compared between larvae fed with control food, or food containing cyclotides (psyleio A, psybry A) solubilized in water. Geometric forms represent different larvae. Statistical differences were analyzed using a one-way ANOVA, followed by a Tukey test: $^{**}p < 0.01$.

Psychotria alkaloids may be advantageous to larvae growth due to antioxidant properties. Treatments containing 2% ethanol were harmful to *H. armigera* larvae, affecting both survival and growth rate (Figure S7), and at least 25% of larvae failed to survive after 72 h of treatment, including control samples, except in the presence of the alkaloids brachycerine or psychollatine (at 5 nmol g^{-1} and 1 µmol g^{-1}). Ethanol is known to cause oxidative stress, resulting in developmental lethality and delay in *Drosophila* (45). Ethanol may also have caused the observed impairment in larvae growth. Although no significant differences were observed when comparing means of relative growth rate among ethanol treatments, when

analyzing the kinetics of growth rate of each individual larva, mass maintenance is observed in control samples. On the other hand, an overall inspection shows that mass increases over time and higher final masses of larvae are visible in plant extracts and alkaloids treatments (Figure 4, Figure S8). In fact, even when the differences were not significant, clear trends were observed. At 48 h, a tendency of weight loss was observed in control ($p = 0.09$) and, after 72 h in GPV presence, a tendency of larvae weight increment ($p = 0.06$) was detectable.

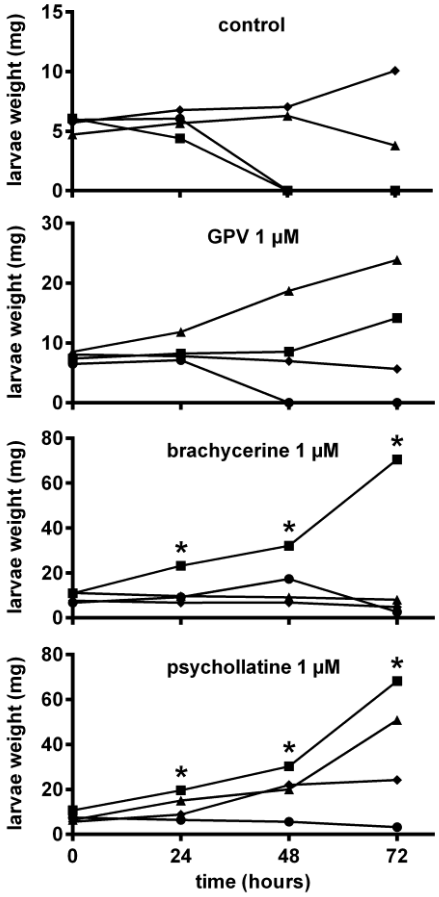

Figure 4. Kinetics of *H. armigera* larvae growth and death in 72 h of feeding trials in ethanol 2% treatments containing the alkaloids brachycerine, GPV, or psychollatine, at 1 µM. Each line in graphs represents one larva. Statistical differences (*) were analyzed by unpaired Student´s t-test against initial time; $p < 0.05$.

These *Psychotria* alkaloids have strong and broad antioxidant properties, possibly helping plants to cope with oxidative stress associated with herbivory and abiotic stress conditions (26). These metabolites could also be acting as reactive oxygen species scavengers, alleviating the toxic effect of ethanol on larvae. Highest levels of alkaloids seemed to be more beneficial to larvae, reflected by increased gain of mass in presence of higher concentrations

of brachycerine and psychollatine. Mortality was reduced from 50% to 25% with higher levels of GPV (Figure 4, Figure S8). Plant extracts also showed similar effects (Figure S8). In the case of extracts, however, it is important to consider that plant extracts contain a range of compounds, including flavonoids, alkaloids, cyclotides, and tannins, which could potentially be harmful or protective to the larvae.

***Response to wounding and defense signaling molecules related to herbivores and pathogens in* P. brachyceras *and* P. leiocarpa**

The plant extract from both evaluated *Psychotria* species are very complex, which made necessary the use of a refined method to monitor specific known compounds. The first step was to isolate target compounds from the respective plants. This is the first work to evaluate changes in cyclotide metabolism under stressful conditions related to wounding and phytohormones associated with herbivory or pathogen responses.

No alteration was observed in cyclotide levels under mechanical wounding, or herbivore and pathogen-related phytohormones (jasmonate and salicylate) (Figure 5), indicating a phytoanticipin-like (*i.e.* constitutive accumulation) profile for these compounds, at least for the five monitored cyclotides in these two *Psychotria* species. No alterations in alkaloid content were observed either (Figure 5).

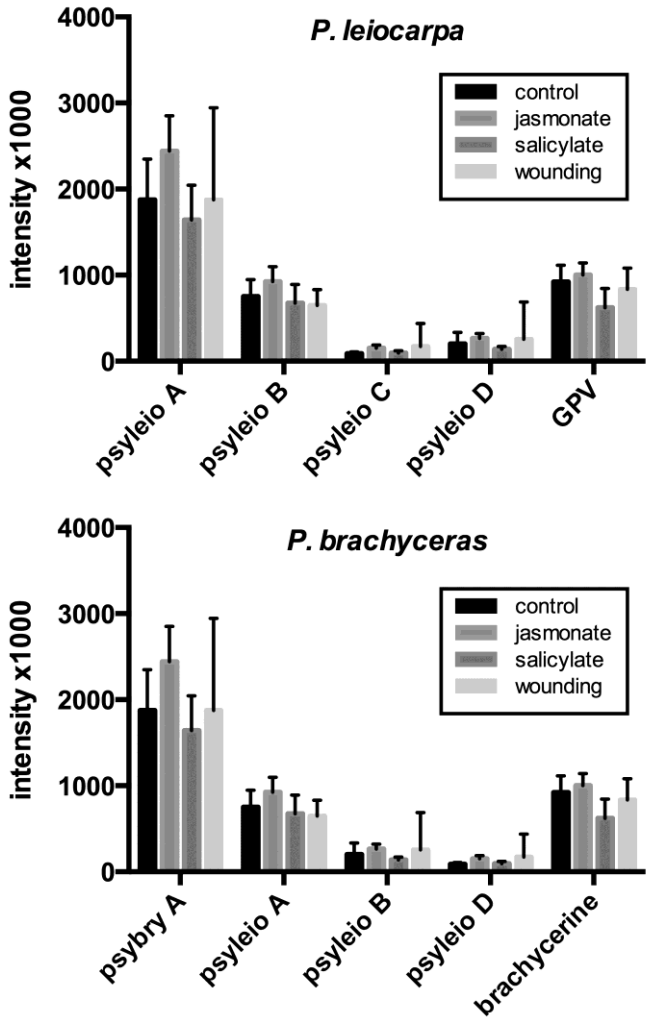

Figure 5. Cyclotides and alkaloid levels in *P. leiocarpa* and *P. brachyceras* leaves after 96 h of treatment (mechanical wounding, jasmonate and salicylate). The four most abundant cyclotides (psyleio A-D) and major alkaloid (GPV) from *P. leiocarpa*, and most abundant cyclotide and alkaloid from *P. brachyceras* (psybry A and brachycerine, respectively) were monitored; additionally, three cyclotides (psyleio A, psyleio B and psyleio D) shared between the two species were also monitored in *P. brachyceras*. Data from extracted ion chromatograms (XIC) of multiple reaction monitoring (MRM) of each sample were normalized using an internal standard (conotoxin) and internal standard curve. No statistical differences were observed in yields between different treatments (one-way ANOVA followed by Tukey test; $p < 0.05$).

GPV had been shown not responsive to the elicitation treatments used in the present work (29), although brachycerine had a similar profile. Brachycerine accumulation has been shown to be increased by wounding and jasmonate treatment (30, 32). The lack of induction in

brachycerine accumulation by wounding could be related to the time sampled in the present experiments (96 h), which has previously been shown to be associated with a return of alkaloid concentration back to control levels, after a peak induction at 48 h after damage infliction (32). The lack of response to jasmonate at the same time point was unexpected, but may reflect differences in the time course induction of brachycerine in whole cuttings versus leaf disks.

Yields obtained by the isolation method and through internal standard curve are in good agreement, showing that the individual concentrations of the major cyclotide from *P. leiocarpa*, psyleio A, and the major cyclotide from *P. brachyceras*, psybry A, are close to 0.1% DW in leaves. Yields of the combined pool of naturally occurring cyclotides may be even more effective in deflecting herbivory attack.

In this study we found a new species containing cyclotides, *P. leiocarpa*, and eight novel cyclotides (Table 2) from two *Psychotria* species, increasing to a total number of 24 the cyclotides discovered in the entire genus.

Table 2. Novel cyclotides in *Psychotria* species (Rubiaceae) and corresponding sequences

Table 2. Novel cyclotides in *Psychotria* species (Rubiaceae) and corresponding sequences

Species	Peptide	Sequence						Mass (Da)*
		Loop	*1*	*2*	*3*	*4*	*5*	*6*	
P. leiocarpa	psyleio A	-G-LPI	C GET C	-FTGT C	--NTPG C	S C	TYP-I C	TRD	3355.23
	psyleio B	-GDIPL	C GET C	-FGGT C	--NTPG C	V C	-AWPV C	NR	3315.23
	psyleio C	-GDIPV	C GET C	-FGGT C	--NTPG C	V C	-AWPV C	TR	3288.22
	psyleio D	-G-LPV	C GES C	-FGGT C	--NTPG C	S C	-TWPV C	TRD	3292.17
	psyleio E	SVTPIV	C GET C	-FGGT C	--NTPG C	S C	-SWPI C	TK	3393.26
P. brachyceras	psyleio A	-G-LPI	C GET C	-FTGT C	--NTPG C	S C	-TYPI C	TRD	3355.23
	psyleio B	-GDIPL	C GET C	-FGGT C	--NTPG C	V C	-AWPV C	NR	3315.23
	psyleio D	-G-LPV	C GES C	-FGGT C	--NTPG C	S C	-TWPV C	TRD	3292.17
	psybry A	-GFNP-	C GET C	IWFPT C	--HAPG C	T C	SIANI C	VRN	3656.41
	psybry B	-GFNP-	C GET C	WNKPT C	--HAPG C	T C	SIANI C	VRN	3638.39
	psybry C	-GFNP-	C GET C	QIDQT C	--HAPG C	T C	SIANI C	VRN	3597.36
	cycloviolacin O17	-G-IP-	C GES C	-VWIP C	ISAAIG C	S C	-KNKV C	YRN	3516.45
O. affinis	kalata B1	-G-LPV	C GET C	-VGGT C	--NTPG C	T C	-SWPV C	TRN	2892.28
	kalata B2	-G-LPV	C GET C	-FGGT C	--NTPG C	S C	-TWPI C	TRD	2955.35

*mass of linear forms of cyclic peptides

Cyclotides of southern Brazilian *Psychotria* species seem to take part in the key role of protecting against herbivores. This could explain the high degree of leaf predation observed in the field for the cyclotide and alkaloid-free *P. carthagenenesis*. Besides, *P. carthagenensis* is

not only pollinated both by exotic and native bee species, but also interacts with a relatively larger spectrum of secondary pollinators (46), which could be due to reduced chemical defenses. A combined strategy with indole alkaloids acting in general defense mechanisms as antioxidants and cyclotides imparting insecticidal properties could be advantageous and may help explaining the much lower leaf predation observed in the field for *P. leiocarpa* and *P. brachyceras*, in spite of and their relatively larger abundance in the understorey of Brazilian Atlantic Forest (35).

P. umbellata proved to be devoid of cyclotides, although it has some of the highest contents of a single indole alkaloid, psychollatine (4% DW in inflorescences) (47), in addition to three derived alkaloids (48). Although insecticidal activity of *P. umbellata* alkaloids remains to be evaluated, it is interesting to note that this species lacking cyclotides has relatively scarce distribution in the understorey. *P. gitingensis* was reported as an alkaloid-free species (49), and if it also proves to be devoid of cyclotides, could be used to evaluate in further detail its interaction with insects, along with similar aspects of *P. carthagenensis*. Screening other *Psychotria* species for the co-occurrence of antioxidant indole alkaloids and cyclotides as a putative (and perhaps widespread) defense strategy against herbivores should be pursued in the future.

Supporting Information

Figure S1. Characterization of novel cyclotides from *Psychotria leiocarpa*. MALDI-TOF/TOF sequencing of the precursor masses 3315.22, 3288.21, 3292.17 and 3393.26 Da from a trypsin digest and an endoproteinase GluC allowed the characterization of the novel cyclotides psyleio B (A), psyleio C (B), psyleio D (C) and psyleio E (D) respectively, based on observed b- and y-ions. All shown masses are monoisotopic.

Figure S2. Characterization of novel cyclotides from *Psychotria brachyceras*. MALDI-TOF/TOF sequencing of the precursor masses 3292.17, 3355.23, 3315.22 and 3392.17 Da from a trypsin digest and an endoproteinase GluC allowed the characterization of the novel cyclotides psybry A (A), psyleio A (B), psyleio B (C) and psyleio D (D) respectively, based on observed b- and y-ions. All shown masses are monoisotopic.

Figure S3. Characterization of novel cyclotides from *Psychotria brachyceras*. MALDI-TOF/TOF sequencing of the precursor masses 3638.39, 3597.36 and 3516.45 Da from a

trypsin digest and an endoproteinase GluC allowed the characterization of the novel cyclotides psybry B (E), psybry C (F) and cycloviolacin O17 (G) respectively, based on observed b- and y-ions. All shown masses are monoisotopic.

Figure S4. MS-MS sequencing after endoproteinase GluC digestion. The *Psychotria* cyclotides psyleio A and psyleio B was sequenced by nanospray MS-MS to support MALDI-TOF/TOF evidence.

Figure S5. MS-MS sequencing after endoproteinase GluC digestion. The *Psychotria* cyclotides psyleio C and psyleio D was sequenced by nanospray MS-MS to support MALDI-TOF/TOF evidence.

Figure S6. MS-MS sequencing after endoproteinase GluC digestion. The *Psychotria* cyclotides psybry A and cycloviolacin O17 was sequenced by nanospray MS-MS to support MALDI-TOF/TOF evidence.

Figure S7. Insecticidal assay. The weight at 24, 48, and 72 h was compared to initial weight. Relative growth after 24, 48, and 72 h was compared between larvae fed with control food, or food containing cyclotide (psyleio B), alkaloids (brachycerine, GPV, psychollatine), or extracts obtained from plants (*P. brachyceras*, *P. carthagenensis*, *P. leiocarpa*, *P. umbellata*) solubilized in ethanol 2%. No statistical differences were found (one-way ANOVA, followed by a Tukey test: $p < 0.05$).

Figure S8. Kinetics of *H. armigera* larvae growth and death in 72 h of feeding trials in ethanol 2% treatments containing alkaloids (brachycerine, GPV, psychollatine; at 5 nM), or alkaloid-bearing plants (*P. brachyceras*, *P. leiocarpa*, *P. umbellata*). Each line in graphs represents one larva. Statistical differences were analyzed by unpaired Student´s t-test against initial time; $p < 0.05$.

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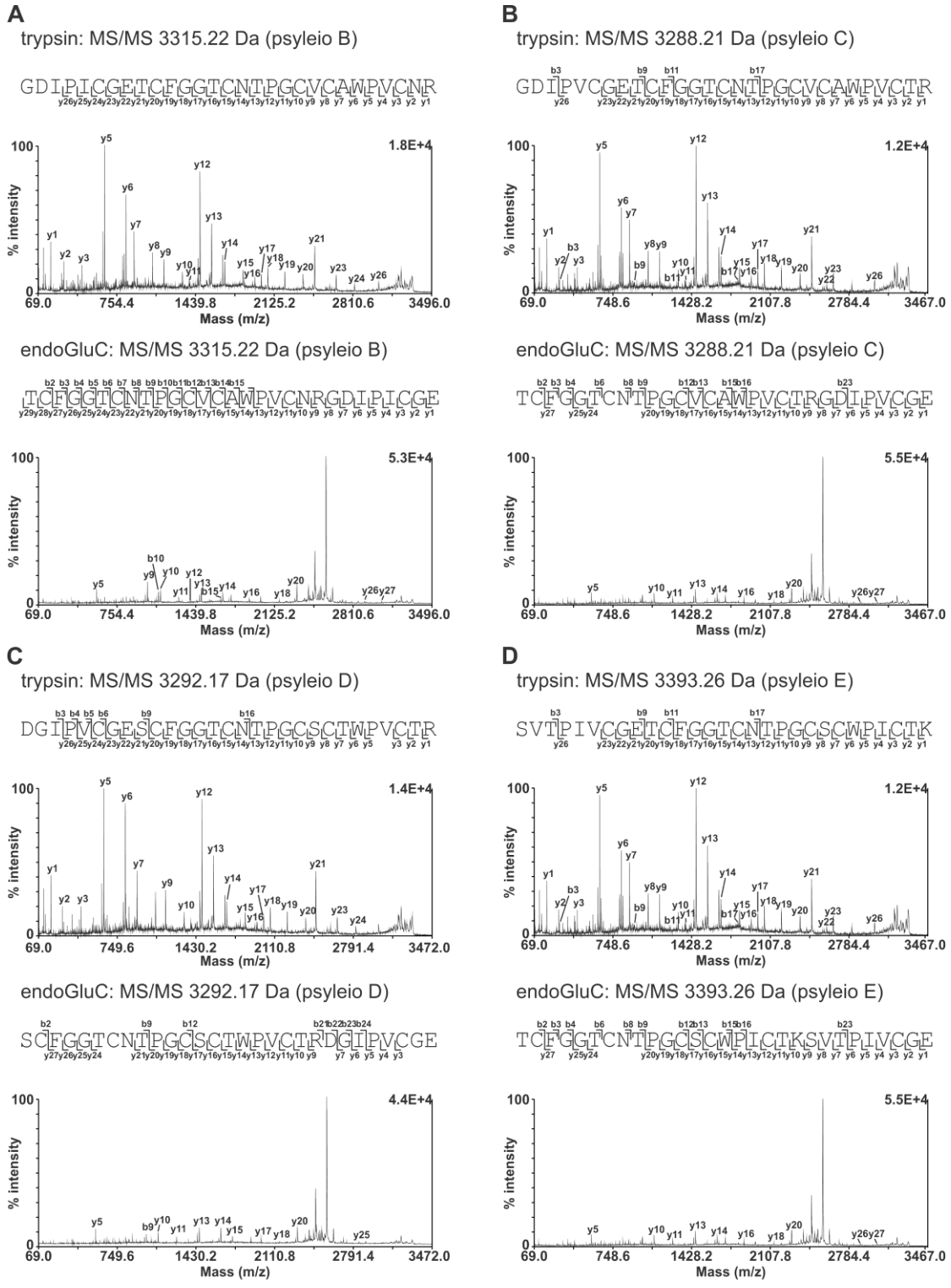
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Figure S1.

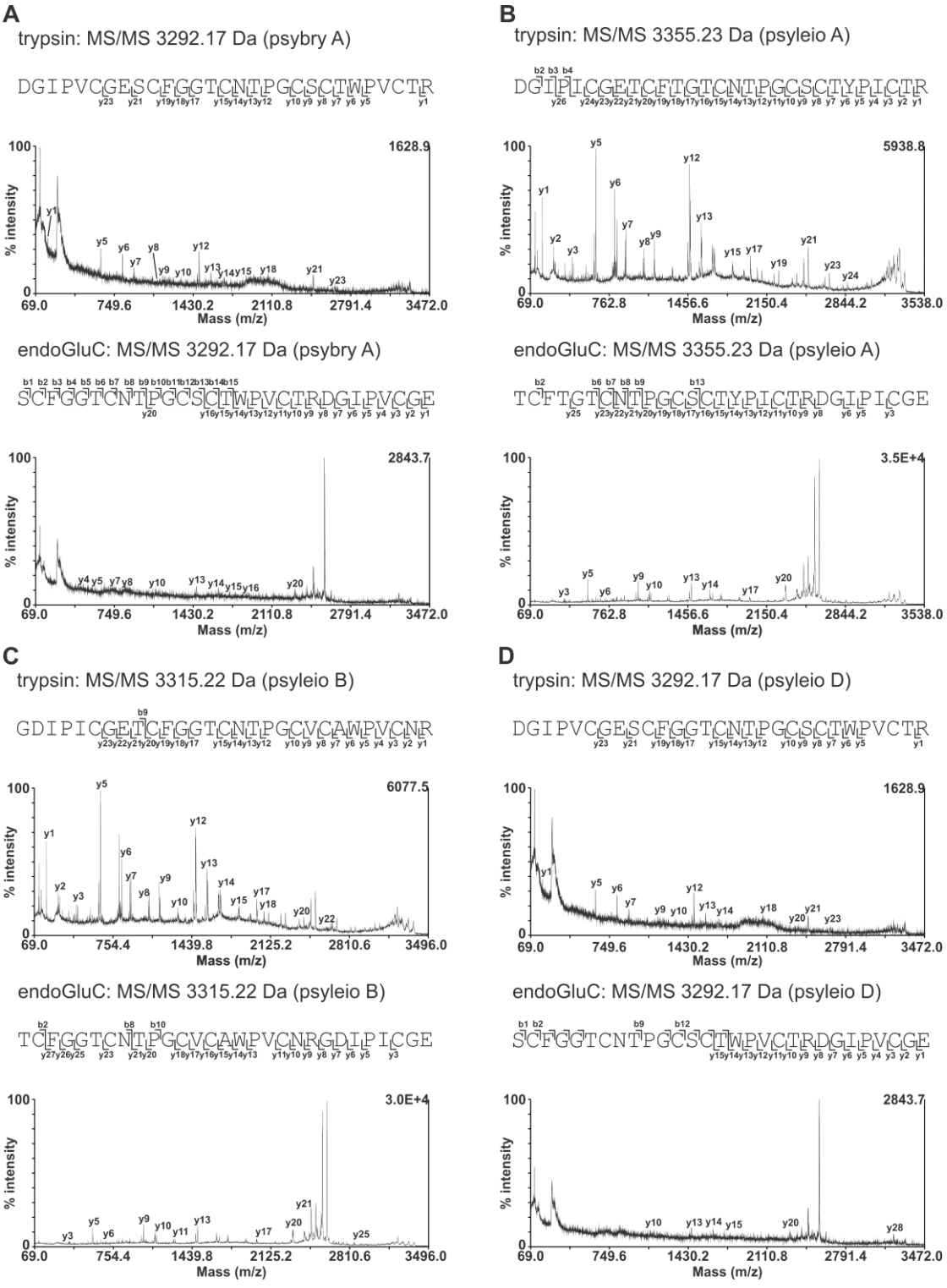
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Figure S2.

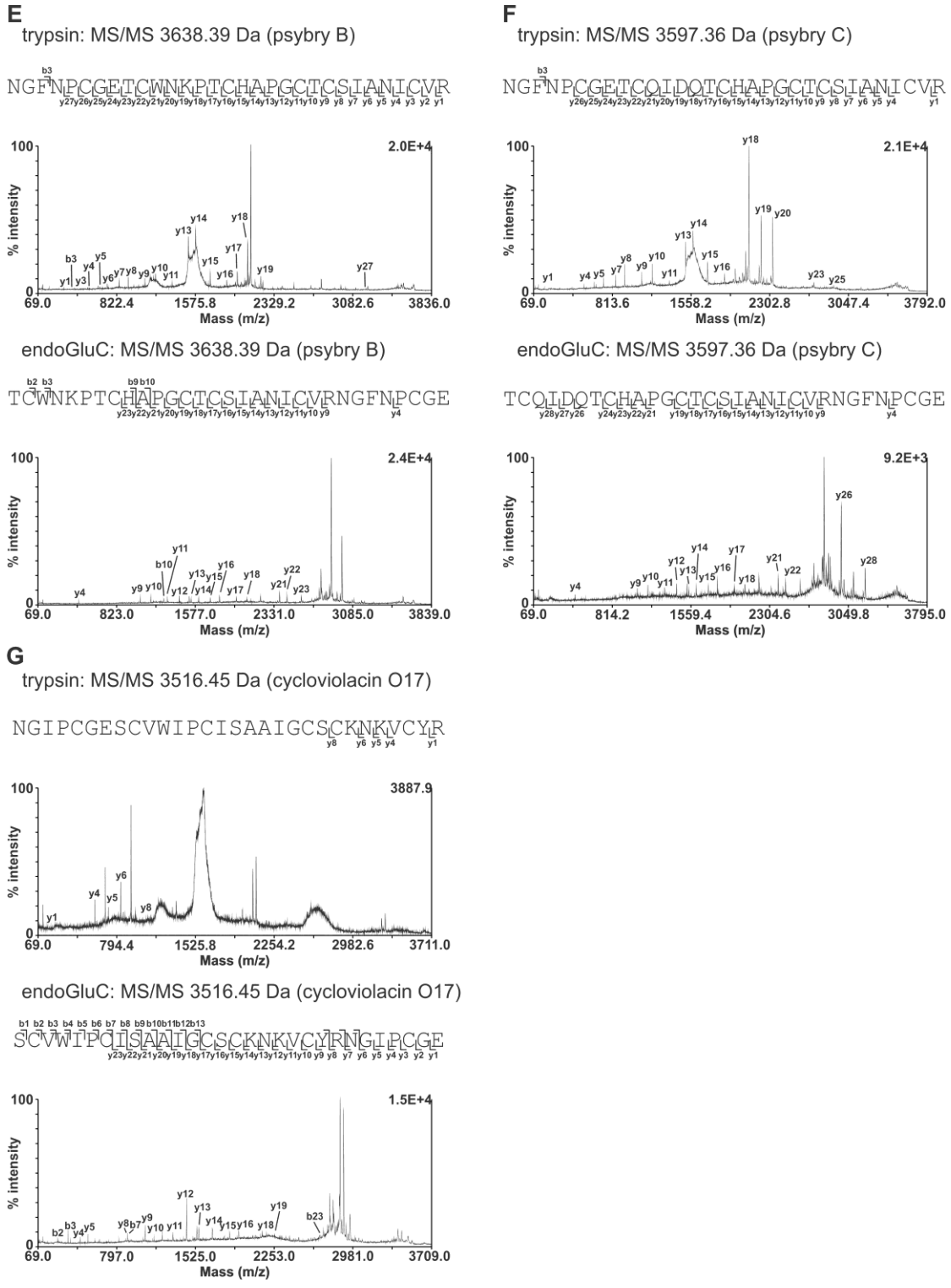
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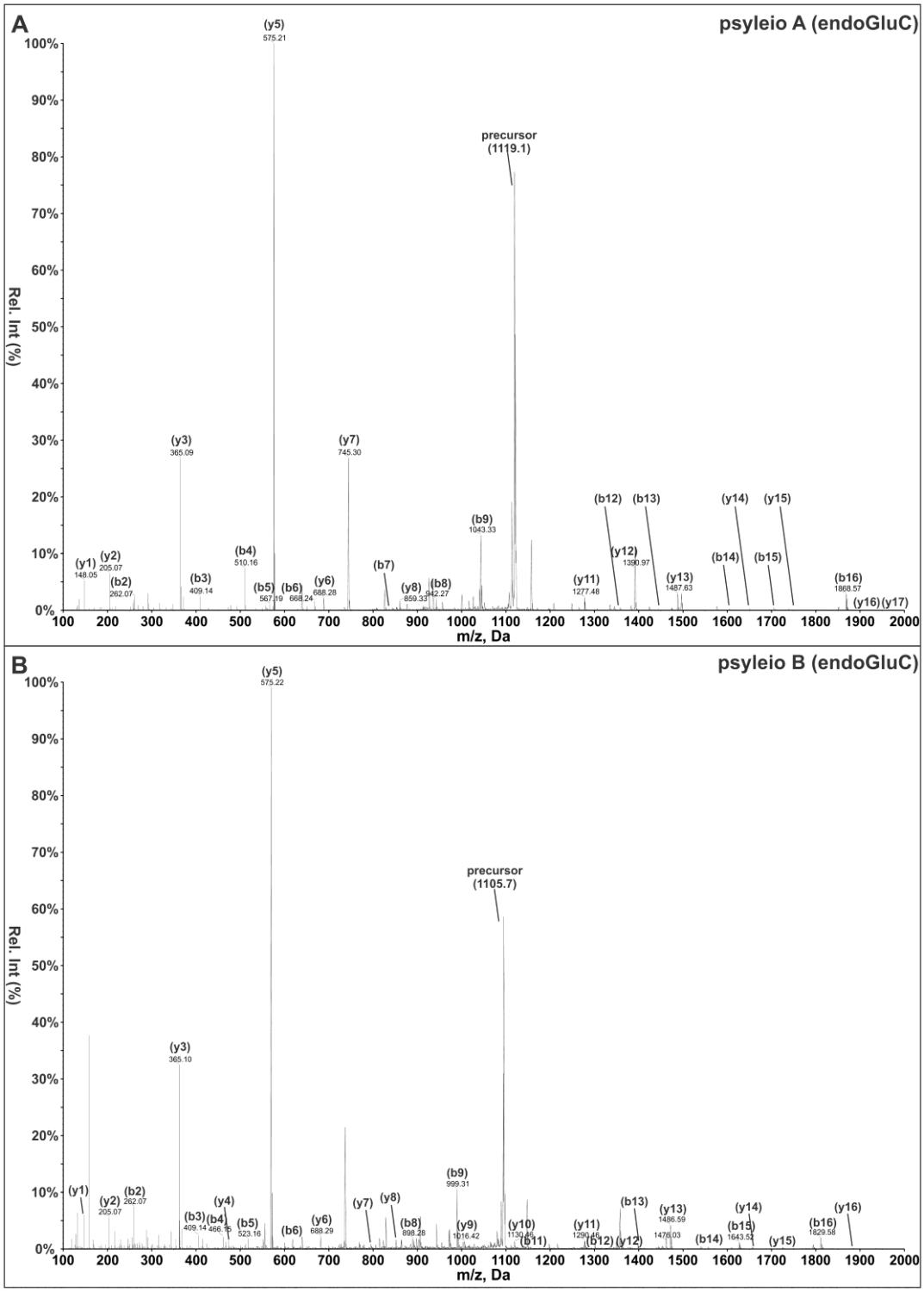
Figure S3.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(y8)
859.33
(b8)
942.27
(y11)
1277.48
(y13)
1487.63
(b16)
1868.57
(y16)
(y17)
Rel. Int (%)
m/z, Da
B
psyleio B (endoGluC)
(y5)
575.22
precursor
(1105.7)
(y3)
365.10
(b9)
999.31
(y1)
(y2)
205.07
(b2)
262.07
(y4)
(b3)
409.14
(b4)
466.15
(b5)
523.16
(b6)
(y6)
688.29
(y7)
(y8)
(b8)
898.28
(y9)
1016.42
(y10)
1130.46
(b11)
(y11)
1290.49
(b12)
(y12)
(b13)
(y13)
1486.59
1476.03
(b14)
(y14)
(b15)
1643.53
(y15)
(b16)
1829.58
(y16)
Rel. Int (%)
m/z, Da

Figure S4.

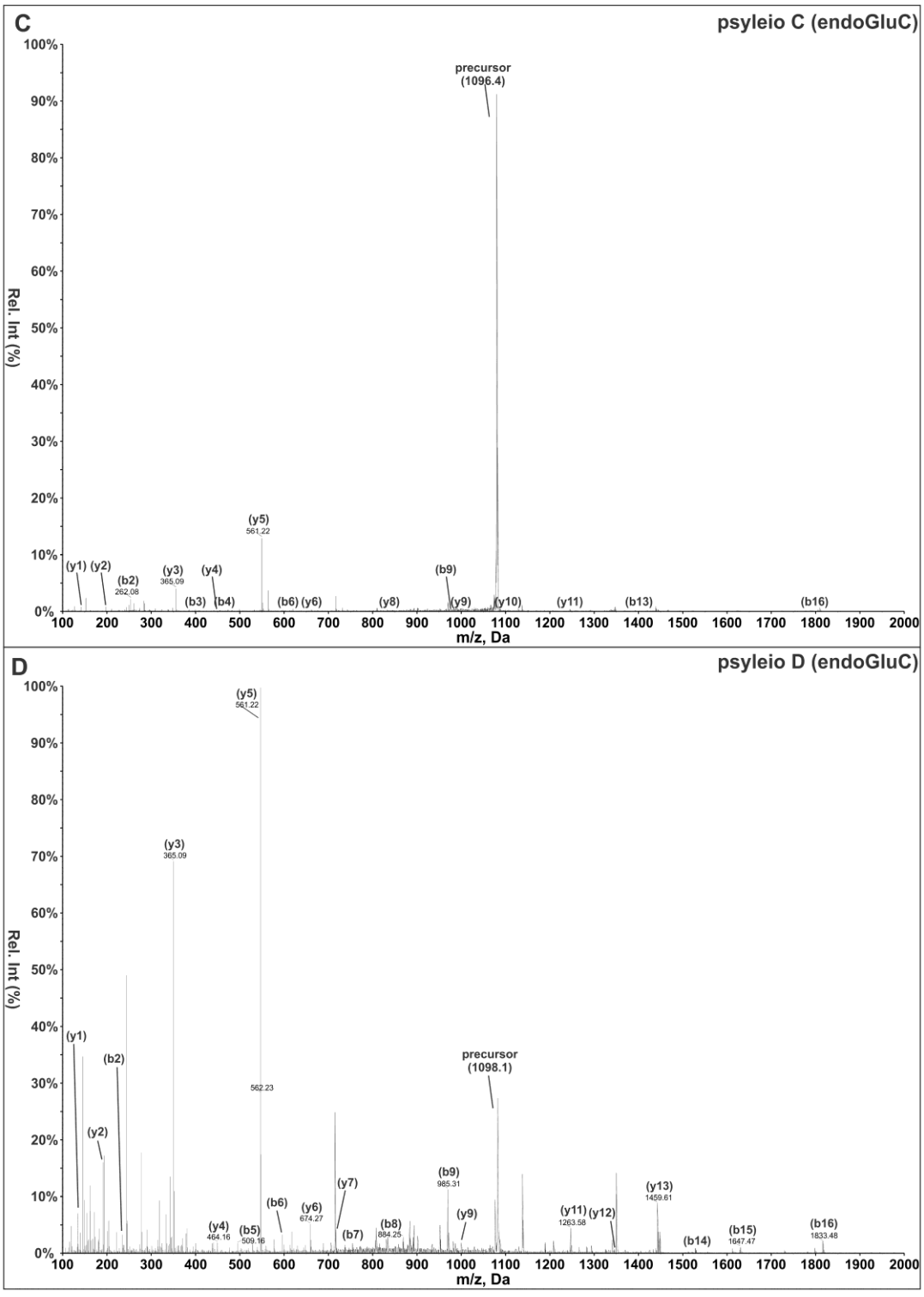
C
psyleio C (endoGluC)
precursor
(1096.4)
(y5)
561.22
(y1)
(y2)
(b2)
262.08
(y3)
365.09
(y4)
(b3)
(b4)
(b6)
(y6)
(y8)
(b9)
(y9)
(y10)
(y11)
(b13)
(b16)
Rel. Int (%)
m/z, Da
D
psyleio D (endoGluC)
(y5)
561.22
(y3)
365.09
(y1)
(b2)
(y2)
562.23
precursor
(1098.1)
(b9)
985.31
(y7)
(b6)
(y6)
674.27
(y4)
464.16
(b5)
509.16
(b7)
(b8)
884.25
(y9)
(y11)
1263.58
(y12)
(y13)
1459.61
(b14)
(b15)
1647.47
(b16)
1833.48
Rel. Int (%)
m/z, Da

Figure S5.

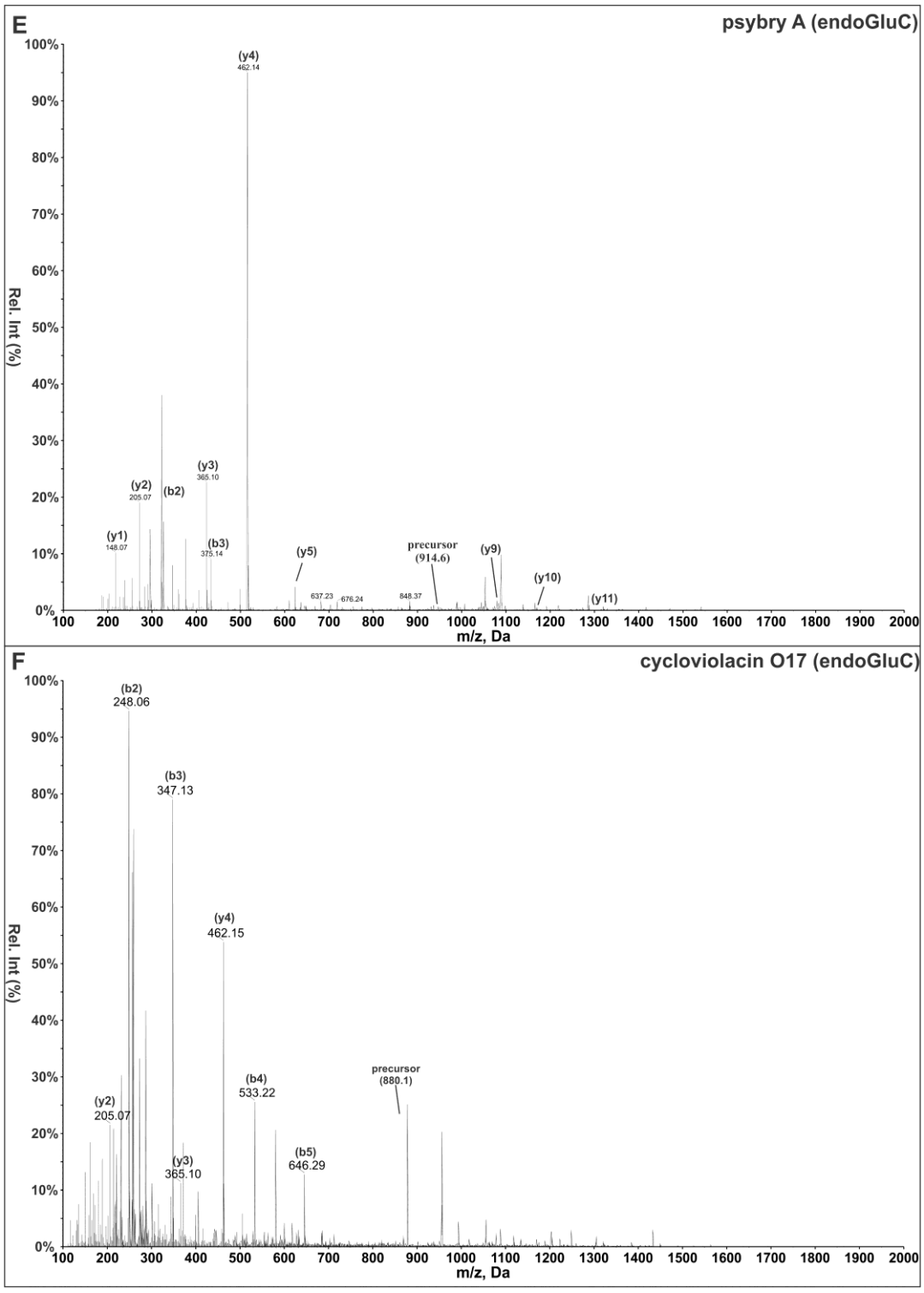
E
psybry A (endoGluC)
Rel. Int (%)
m/z, Da
(y4)
462.14
(y2)
205.07
(b2)
(y3)
365.10
(y1)
148.07
(b3)
375.14
(y5)
637.23
676.24
precursor
(914.6)
848.37
(y9)
(y10)
(y11)
F
cycloviolacin O17 (endoGluC)
Rel. Int (%)
m/z, Da
(b2)
248.06
(b3)
347.13
(y4)
462.15
(b4)
533.22
precursor
(880.1)
(y2)
205.07
(y3)
365.10
(b5)
646.29

Figure S6.

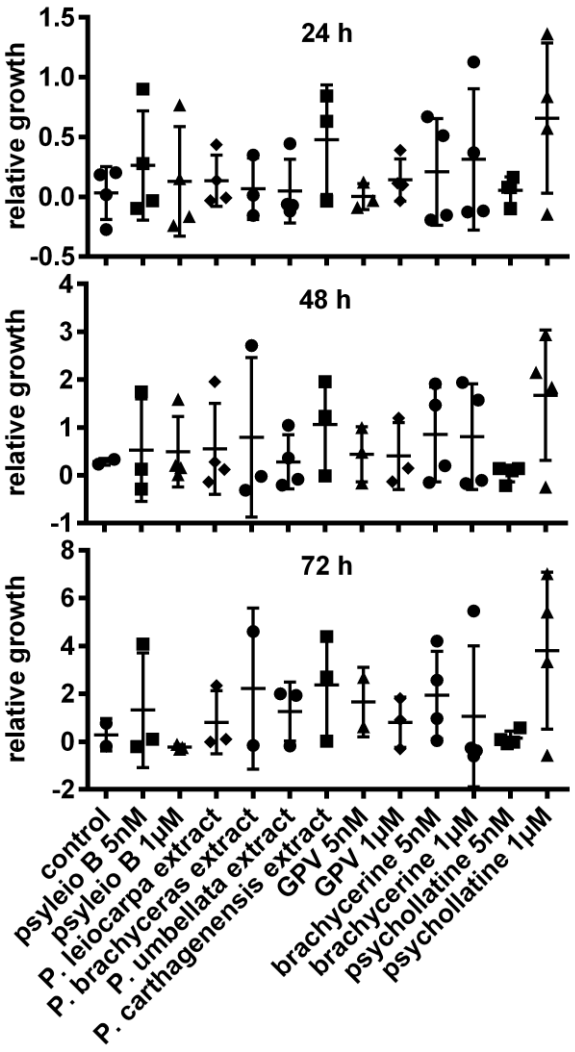
24 h
48 h
72 h
relative growth
1.5
1.0
0.5
0.0
-0.5
4
3
2
1
0
-1
8
6
4
2
0
-2
control
psyleio B 5nM
psyleio B 1µM
P. leiocarpa extract
P. brachyceras extract
P. umbellata extract
P. carthagenensis extract
GPV 5nM
GPV 1µM
brachycerine 5nM
brachycerine 1µM
psychollatine 5nM
psychollatine 1µM

Figure S7.

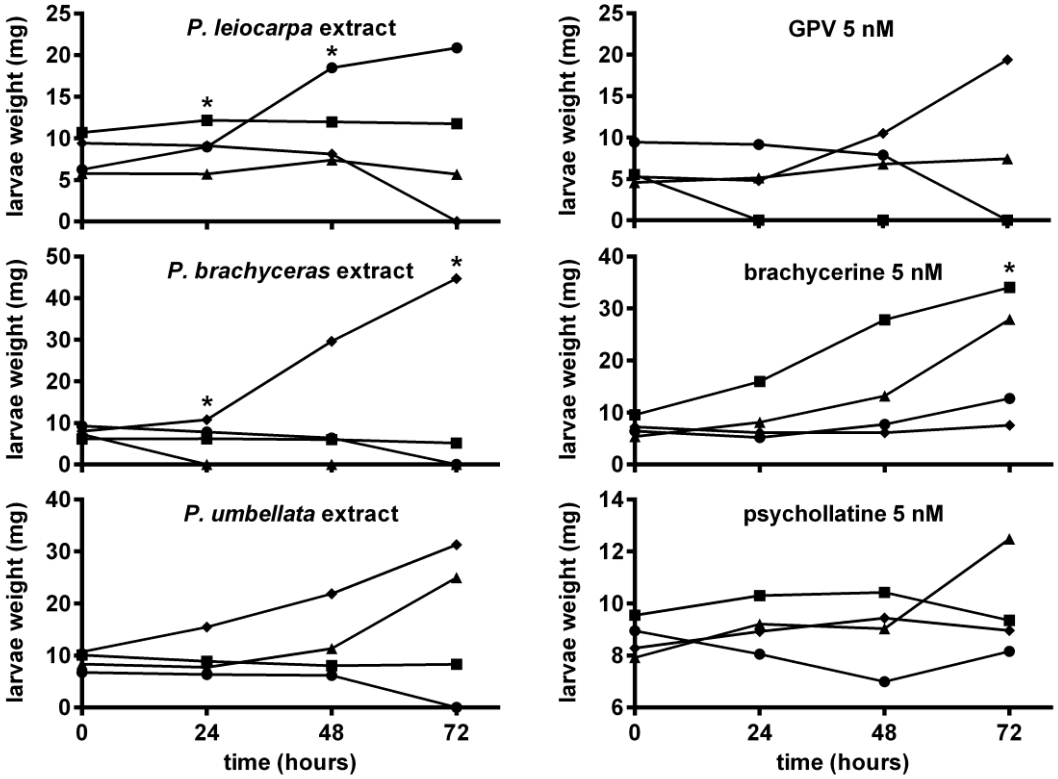
P. leiocarpa extract
GPV 5 nM
P. brachyceras extract
brachycerine 5 nM
P. umbellata extract
psychollatine 5 nM
larvae weight (mg)
time (hours)
0
24
48
72

Figure S8.

Considerações Finais

Os resultados descritos nesta Tese representam uma contribuição útil para a compreensão do metabolismo de alcalóides e ciclotídeos de espécies de *Psychotria* do Sul do Brasil e englobam diversas descobertas inéditas na literatura. Destacam-se a descoberta de oito novos ciclotídeos, bem como a identificação de *Psychotria leiocarpa* como uma espécie produtora de ciclotídeos e *P. umbellata* como aparente não produtora. Por outro lado, é proposto um modelo de ação conjunta de alcalóides e ciclotídeos na defesa contra herbívoros, os primeiros tendo um papel de moduladores do estresse oxidativo e os segundos, efeito inseticida.

Avaliando os principais alcalóides de *P. brachyceras*, *P leiocarpa* e *P. umbellata*, verificamos que os mesmos não parecem apresentar efeito de proteção contra insetos ou gastrópodes, e que apresentam uma ampla e forte ação antioxidante em ensaios *in vitro* (Porto *et al.*, 2014; Matsuura et al, 2013; Nascimento *et al.*, 2013 b). Em tempos mais curtos de exposição a superóxido, os alcalóides apresentam uma eficácia comparável ou maior que a de compostos antioxidantes conhecidos, como vitamina E, e como clássicos agentes antioxidantes de plantas, como o flavonóide rutina (Rau *et al.*, 2014; dados não publicados). Em tempos maiores de exposição, ocorre um declínio nas propriedades antioxidantes dos alcalóides, bem como da vitamina E, mas não de rutina (Figura 4, a metodologia e as amostras foram as mesmas descritas em Rau *et al.*, 2014).

Devido ao fato de ocorrerem elevadas concentrações de alcalóides indólicos nessas espécies, constitutivos, ou no caso da braquicerina, elicitáveis sob estresses de naturezas distintas (Gregianini *et al.*, 2004; Gregianini *et al.*, 2003), é provável que a presença dos alcalóides antioxidantes atue auxiliando na detoxificação das espécies reativas de oxigênio. Pode-se conjecturar um modo de ação em que os alcalóides mitigariam as espécies reativas geradas em excesso num primeiro momento de distúrbio e, a partir daí, a ação dos flavonóides assumiria maior importância para evitar danos à planta.

O acúmulo desses alcalóides parece estar fortemente relacionado a situações que requerem uma maior proteção oxidativa. Exemplos incluem os comportamentos observados para braquicerina de *P. brachyceras* (Gregianini *et al.*, 2003) e para GPV de *P. leiocarpa* (Henriques *et al.*, 2004), presentes em estruturas mais sensíveis e mais expostas à luz, e durante o desenvolvimento, passíveis de indução em folhas mais jovens, o que poderia estar relacionado a um balanço entre crescimento/defesa.

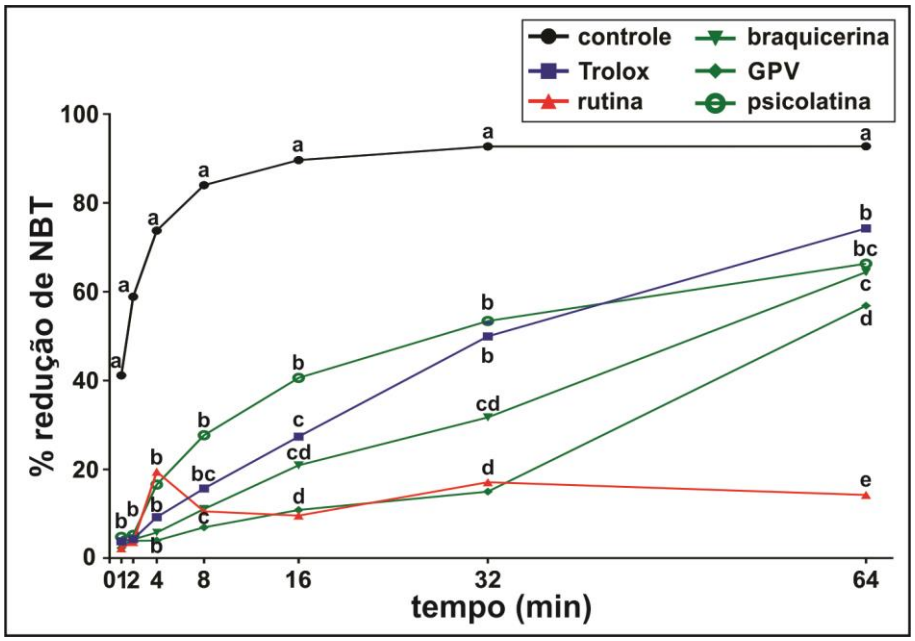

Figura 4. Ensaio antioxidante *in vitro* comparando o potencial antioxidante de alcalóides indólicos (braquicerina, GPV e psicolatina; em verde), flavonóide rutina (vermelho), e um análogo de vitamina E, Trolox™ (azul), contra radicais superóxido (% redução de *nitro blue tetrazolium*), após 1, 2, 4, 8, 16, 32 e 64 minutos de reação. Em todos ensaios a concentração dos compostos foi 5 mM; nas amostras controle (preto), não houve a presença de nenhum composto adicional durante a reação de redução. As comparações estatísticas só são validas entre diferentes compostos em um mesmo tempo de reação (ANOVA seguido de Tukey, $p < 0,05$).

Em relação aos ciclotídeos, este foi o primeiro trabalho a monitorar os teores de ciclotídeos após tratamentos relacionados à herbivoria (simulado por dano mecânico e aplicação de jasmonato) e na presença de patógenos (simulado por salicilato). Não houve alteração do teor de ciclotídeos avaliados após 96 h de tratamento nas folhas de *P. brachyceras* e *P. leiocarpa*, indicando um padrão de acúmulo constitutivo.

Este trabalho também contribuiu para o conhecimento da diversidade de ciclotídeos no gênero *Psychotria*. Foram constatadas uma nova espécie produtora de ciclotídeos, *P. leiocarpa*, e uma nova espécie não-produtora, *P. umbellata*. Até o presente momento, apenas 16 ciclotídeos foram descritos em espécies de *Psychotria* (Witherup *et al.*, 1994; Gruber *et al.*, 2008; Gerlach *et al.*, 2010; Koehbach *et al.*, 2013; Hellinger *et al.*, 2015) e 6 ciclotídeos foram descritos na antiga *P. ipecacuanha* (atualmente *Carapichea ipecacuanha*) (Koehbach *et al.*, 2013). No presente trabalho, constatou-se a existência de oito ciclotídeos inéditos no gênero *Psychotria*. Além disso, foi confirmada a ausência de ciclotídeos em *P. carthagenensis*

(Gruber *et al.*, 2008; Gerlach *et al.*, 2013). Esta mesma espécie não apresenta alcalóides nas populações do Rio Grande do Sul (Leal e Elizabetsky, 1996), embora existam relatos da presença de alcalóides em espécimes de regiões mais ao Norte do Brasil que são, por vezes, utilizados como substitutos de *P. viridis* no preparo da bebida alucinogênica Ayahuasca (Rivier e Lindgren, 1972). Nos trabalhos aqui realizados, *P. carthagenensis* foi comparada com outras espécies de *Psychotria* e utilizada como uma forma de "controle negativo" de alcalóides e ciclotídeos.

O ciclotídeo psyleio A, com atividade inseticida, foi detectado em *P. brachyceras* e *P. leiocarpa*, o que pode ajudar a explicar, ao menos em parte, a menor taxa de predação observada nessas espécies em relação à *P. carthagenensis*, espécie deficiente em alcalóides e ciclotídeos. Psyleio A é o ciclotídeo majoritário de *P. leiocarpa* atingindo teores próximos a 0,1% do peso seco de suas folhas; em *P. brachyceras*, seu teor é menor, porém ainda relevante (aproximadamente 0,05% do peso seco). Adicionalmente, esta última espécie apresenta 2 ciclotídeos descritos no presente trabalho ainda a serem testados, além de um *pool* de pelo menos 11 ciclotídeos não caracterizados. A deficiência de *P. carthagenensis* em manter uma defesa química evidente parece afetar o acesso de insetos de uma forma geral, tendo como aspecto favorável a ocorrência de um maior espectro de polinizadores compatíveis (Mesquita-Neto *et al.*, 2015).

P. umbellata não parece apresentar ciclotídeos e, embora de ocorrência bem mais esparsa e com menor porte, também aparenta ser menos predada quando comparada com *P. carthagenensis*. Este perfil poderia estar ligado ao fato desta espécie apresentar as maiores concentrações de alcalóides dentre as estudadas, destinando um alto investimento de seus recursos energéticos. O alcalóide majoritário de *P. umbellata*, psicolatina, assim como os alcalóides indólicos de outras espécies de *Psychotria* avaliadas, não apresentou toxidez evidente contra insetos. Os teores de psicolatina em *P. umbellata* são extremamente altos, chegando a 4% peso seco em folhas (Paranhos *et al.*, 2005; Paranhos *et al.*, 2009). Recentemente, foram descobertos três novos alcalóides indólicos em *P. umbellata*, todos derivados de psicolatina (Kerber *et al.*, 2014). Estudos mais detalhados são necessários para avaliar a presença de outras classes de alcalóides, bem como as bioatividades dos alcalóides presentes nesta planta, incluindo possível toxidez contra herbívoros.

Por fim, também seria interessante confirmar em outras espécies de *Psychotria* a relação entre manutenção de compostos de defesa e uma maior proteção direta contra predadores. Esforços poderiam ser dirigidos para avaliar a taxa de predação de *P. gitingensis*,

outra espécie de *Psychotria* deficiente em alcalóides (Tan *et al.*, 2012), e também avaliar a presença de ciclotídeos nessas espécies.

A estratégia combinada, observada em *P. brachyceras* e *P. leiocarpa*, de acumular ciclotídeos e alcalóides, possivelmente resulta em eficiente defesa contra predadores em conjunto com sistemas antioxidantes moduladores de estresse oxidativo (Figura 5). Esta combinação defensiva poderia, ao menos em parte, contribuir para a significativa abundância dessas duas espécies em fragmentos da Floresta Atlântica (Müller *et al.*, 2012). Claro está que não é possível excluir a influência de outros metabólitos e estratégias, além de alcaloides e ciclotídeos, na defesa destas plantas contra herbívoros e sua distribuição em florestas. Futuras investigações poderão auxiliar no detalhamento e eventual adição de novas peças do complexo aparato de defesa de *Psychotria* spp.

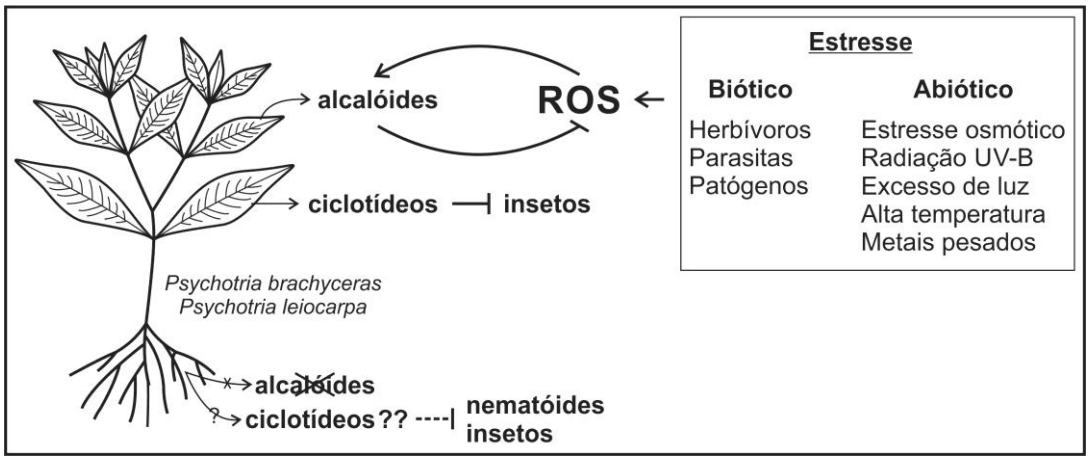

Figura 5. Modelo hipotético da ação de alcalóides e ciclotídeos na proteção contra estresses bióticos e abióticos.

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Anexo I: Capítulo de livro publicado durante o período de Doutorado; submetido no período de Mestrado.

Photoelicitation of bioactive secondary metabolites by ultraviolet radiation: mechanisms, strategies and applications

Livro: Biotechnology for Medicinal Plants- Micropropagation and Improvement
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Editores: S. Chandra, H. Lata e A. Varma

Chapter 7
Photoelicitation of Bioactive Secondary Metabolites by Ultraviolet Radiation: Mechanisms, Strategies, and Applications

Hélio Nitta Matsuura, Fernanda de Costa, Anna Carolina Alves Yendo and Arthur Germano Fett-Neto

7.1 Introduction

UV radiation is divided into three classes: UV-C, UV-B, and UV-A. Although the highly energetic UV-C (200–280 nm) is completely absorbed by atmospheric gases and UV-A (315–400 nm) is hardly absorbed by ozone, the potentially harmful UV-B (280–320 nm) is only partially absorbed by atmospheric ozone, comprising approximately 4% of terrestrial radiation. In the last 20 years, the depletion of the stratospheric ozone layer, catalyzed by chlorofluorocarbons and other pollutants, resulted in rising levels of the sun's UV-B radiation reaching the Earth's surface. Due to the high energy of UV-B radiation, even modest increases could lead to significant biological damage (Jansen et al. 1998; Frohnmeyer and Staiger 2003).

Elevations of UV-B radiation levels have effects on plant development, morphology, and physiology. Such responses include inhibition of plant growth rates, biomass reduction, increased accumulation of UV absorbing secondary metabolites, and influence on numerous ecological processes. In addition to indirect changes, caused by affecting host plant quality, predators, and pathogens,

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Anexo II: Capítulo de livro publicado durante o período de Doutorado; submetido no período de Mestrado.

Bioactive alkaloids from South American *Psychotria* and related Rubiaceae

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Editores: K.G. Ramawat, J.M. Merillon e M. Henry

Bioactive Alkaloids from South American *Psychotria* and Related Rubiaceae

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Hélio Nitta Matsuura, Diogo Denardi Porto
and Arthur Germano Fett-Neto

Contents

Abstract

The largest fraction of global plant diversity is located in the Neotropics, with the Atlantic Forest and the Amazon being a rich untapped reservoir of species that may lead to new drug discovery. Bioactive molecules are often isolated from Rubiaceae species. Ethnobotanic and chemotaxonomic studies may provide clues to guide the prospection of bioactive molecules of interest. In South America, three genera are of special interest due to the bioactivities of their phytochemicals along with their importance to local human populations: *Uncaria*, *Cinchona*, and *Psychotria*. The numerous bioactivities of alkaloids from species in these genera

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Anexo III: Artigo publicado durante o período de Doutorado; submetido no período de Mestrado.

The major indole alkaloid N,*β*-D-glucopyranosyl vincosamide from leaves of *Psychotria leiocarpa* Cham. & Schltdl. is not an antifeedant but shows broad antioxidant activity

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The major indole alkaloid N,β-D-glucopyranosyl vincosamide from leaves of *Psychotria leiocarpa* Cham. & Schltdl. is not an antifeedant but shows broad antioxidant activity†

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N,β-D-glucopyranosyl vincosamide (GPV), a major alkaloid of *Psychotria leiocarpa*, constitutes up to 2.5% of the dry weight in leaves. Alkaloid content was not elicited by mechanical wounding or jasmonate. At concentrations found in natural conditions or 2.5 fold higher, GPV did not inhibit herbivory in two unrelated generalist models (*Helix aspersa* and *Spodoptera frugiperda*) or in a specific interaction model (*Heliconius erato* fed with *Passiflora suberosa*). *In situ* staining assay showed quenching activity of hydrogen peroxide by GPV. Exposure of *P. leiocarpa* to acute UV-B stress did not change GPV or chlorophyll content, indicating high tolerance to this stress by the species. *In vitro* antioxidant tests against singlet oxygen, superoxide anions and hydroxyl radicals showed efficient quenching activity of the alkaloid. GPV was not effective as antifeedant, but it may act indirectly in *P. leiocarpa* protection against oxidative stress generated upon wounding, UV exposure and perhaps other environmental stresses.

Keywords: monoterpene indole alkaloid (MIA); herbivory; wounding; jasmonate; phytoanticipin; UV-B radiation; reactive oxygen species

1. Introduction

Psychotria is the largest genus of Rubiaceae and Neotropical species have been found to accumulate indole alkaloids, some of which display relevant pharmacological properties (Elisabetsky et al., 1997), including analgesic, anti-inflammatory, anxiolytic, antidepressant and antioxidant effects (Both, Kerber, Henriques, & Elisabetsky, 2002; Elisabetsky et al., 1997; Fragoso et al., 2008; Nascimento et al., 2007). *Psychotria leiocarpa* is an understorey woody species from Southern Brazil capable of accumulating alkaloids. The monoterpene indole alkaloid N,β-D-glucopyranosyl vincosamide (GPV) (Figure 1) is the major alkaloid in the leaves of this species, reaching concentrations of up to 2.5% dry weight (DW) (Henriques et al., 2004).

Secondary metabolites may play a major role in plant defence, and direct defences involve repellent or anti-digestive action, reported for alkaloids, phenolics, glucosinolates, cyanogenic glucosides and proteinase inhibitors (Bonaventure & Baldwin, 2010). Also,

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†This article is dedicated to Prof. Dr Atta-ur-Rahman (University of Karachi), outstanding scientist in the field of natural products research, on the occasion of his 70th birthday.

Anexo IV: Artigo publicado durante o período de Doutorado.

Shoot accumulation kinetics and effects on herbivores of the wound-induced antioxidant indole alkaloid brachycerine of *Psychotria brachyceras*

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Shoot Accumulation Kinetics and Effects on Herbivores of the Wound-Induced Antioxidant Indole Alkaloid Brachycerine of *Psychotria brachyceras*

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A major shoot-specific monoterpene indole alkaloid produced by *Psychotria brachyceras*, brachycerine, is regulated by either wounding or jasmonate application. Highest concentrations of the alkaloid are found in inflorescences, suggesting a defence role. Brachycerine has antimutagenic and antioxidant properties, 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 to leaves increased the content of brachycerine locally. Wounding did not affect phenolics content in *P. brachyceras* leaves, and no tannins were detected in the species. In generalist herbivore bioassays, neither brachycerine nor *P. brachyceras* extracts showed toxic effects. *In vivo* hydrogen peroxide staining assay showed less wound-generated peroxide accumulation in alkaloid treated tissues. This pattern was confirmed in quantitative assays measuring tissue hydrogen peroxide concentrations. Data indicate that brachycerine is not a herbivore deterrent, but rather an indirect chemical defence, modulating oxidative stress caused by mechanical damage.

Keywords: Monoterpene indole alkaloid (MIA), Herbivory, Wounding, Phytoalexin, Hydrogen peroxide, Antioxidant.

The concept of co-evolution and the understanding of plant-insect interactions by an arms race metaphor have provided explanations for a multitude of ecochemical phenomena [1]. Indirect plant defences involve attraction of herbivore predators, which may be mediated by volatile organic compounds [2], whereas direct plant defences are traits that affect herbivore feeding behavior and/or performance [3].

Alkaloids are small nitrogen-containing molecules, often derived from amino acids, whose production and storage by plants are well documented as defence responses to biotic stress [4]. Phenolics, another class of secondary metabolites, are a large group of substances with diverse functions in plants [5]. Among phenolics, tannins are characterized by protein binding and precipitation capacity, being effective in protection against herbivores [6a-b].

Another strategy developed by plants to cope with herbivory stress is tolerance [3]. Mechanical wounding associated with folivory elicits the production of H_2O_2 [7], which, if left unchecked, may cause disruption of cell structures. Accumulation of antioxidant compounds, such as anthocyanins, may alleviate the wound-generated oxidative stress [8], contributing to plant tolerance.

Psychotria brachyceras Mull. Arg. (Rubiaceae) accumulates brachycerine, a monoterpene indole alkaloid, in aerial parts [9]. This alkaloid shows antioxidant and antimutagenic properties and its concentration in leaves is increased by ultraviolet radiation, wounding, and jasmonate exposure [10a-c]. Experiments were carried out to examine putative ecochemical roles of brachycerine with respect to wounding and herbivory. *P. carthagenensis* Jacq., a syntopic and closely related species devoid of alkaloids [11], was examined for comparison.

Plants are exposed to mechanical injury from different sources, including herbivore feeding, animal movement, action of wind and storms. Metabolic changes after mechanical injury may influence responses involved in wound healing and/or prevention against further attacks. Among the possible responses, local and/or systemic accumulation of defence-related secondary metabolites is often observed. Brachycerine concentration in leaves increased in damaged samples and peaked 48 hours after wounding (Figure 1A) [10b]. Significant increases in brachycerine contents were seen from 24 hours after wounding onwards, although a transient increase in alkaloid was also observed 2 hours after leaf damage (Figure 1A). Jasmonate also promotes brachycerine content, being possibly implicated in signalling [10b]. The same kind of regulation can be found in herbivore-deterrent alkaloids, such as nicotine [12], and also in phenolics, such as tannins [13].

Toxic alkaloids such as nicotine display a whole-plant induction upon wounding [14]. Brachycerine contents in stem samples, however, were not changed by leaf wounding (Figure 1B). This result is consistent with a locally restricted induction of the alkaloid reported in leaves [10b].

P. brachyceras leaf samples were assayed for soluble tannins and the extract had no protein precipitation activity; protein-bound and fiber-bound condensed tannins could not be detected. Since no tannins were found in *P. brachyceras* leaves, and no alterations were detected in total phenolics (see Figure S1 – in the supplementary data), brachycerine was thought to be a major