Acta Botanica Brasilica - 33(4): 607-617. October-December 2019.
doi: 10.1590/0102-33062019abb0114

Review

Sustainable production of bioactive alkaloids in *Psychotria* L. of southern Brazil: propagation and elicitation strategies

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Received: April 1, 2019
Accepted: June 28, 2019

ABSTRACT

Psychotria is the largest genus in Rubiaceae. South American species of the genus are promising sources of natural products, mostly due to bioactive monoterpene indole alkaloids they accumulate. These alkaloids can have analgesic, antimutagenic, and antioxidant activities in different experimental models, among other pharmacological properties of interest. Propagation of genotypes with relevant pharmaceutical interest is important for obtaining natural products in a sustainable and standardized fashion. Besides the clonal propagation of elite individuals, the alkaloid content of *Psychotria* spp. can also be increased by applying moderate stressors or stress-signaling molecules. This review explores advances in research on methods for plant propagation and elicitation techniques for obtaining bioactive alkaloids from *Psychotria* spp. of the South Region of Brazil.

Keywords: abiotic stress, alkaloids, elicitation, monoterpenes, plant propagation, *Psychotria*, southern Brazil, sustainability

Introduction

Psychotria belongs to Rubiaceae, one of the major families of flowering plants having economic interest. The family includes coffee, a few significant poisonous plants to livestock, besides several important ornamental and medicinal species (Souza & Lorenzi 2012). *Psychotria* has captured researchers' attention mostly because of its medicinal properties.

Psychotria colorata is an Amazonian species that produces polyindolinic alkaloids with analgesic activity (Matsuura *et al*. 2013). The promising results obtained with *P. colorata* motivated the investigation of southern Brazilian *Psychotria* species and the discovery of new bioactive alkaloids (Porto *et al*. 2009). Moreover, leads on *in planta* alkaloid functions were also topic of experimental evaluation.

One of the key elements that needs to be addressed early on during the process of developing new bioactive molecules from plants is the capacity to generate catalytically active biomass to support extraction and steady supply. There are a number of ways through which these goals may be reached, including greenhouse rooting of cuttings (mini-cutting

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system), in vitro organ, whole plants, somatic embryos, or cell cultures, and even transfer of metabolic pathways to heterologous systems (*e.g.* yeast and bacteria) (Matsuura *et al.* 2018). The best approach to be taken must be examined on a case by case basis, since this depends on factors such as growth rates, metabolic activity, concentration of bioactive principles, costs of production, and product value. In case plants are chosen as sources, clonal propagation of some selected genotypes are most useful, since they provide a fast way to achieve genetic improvement, especially for woody plants, and facilitate extraction procedures by increasing homogeneity of metabolic product yields.

Besides appropriate propagation protocols, increased yields of specialized metabolites, including alkaloids, may be obtained using elicitation strategies. In a broad sense, most strategies to elicit higher yields of target metabolites obtained from medicinal plants involve transient exposure to moderate intensity stress, both biotic and/or abiotic. Abiotic treatments comprise exposure to water stress, heat, cold, UV, high irradiance, salt, cell compatible osmotic agents, heavy metals, wounding, ultrasound, mechanical stress, among others. Biotic stress simulates herbivory or pathogen attack, either by wounding or by chemical signals, often the phytohormones jasmonic acid or salicylic acid, as well as ethylene and ABA, which may also mimic several abiotic stresses. The use of signaling molecules, such as phytohormones and hydrogen peroxide, has the advantage of triggering stronger responses, since they are often hubs to which transduction pathways converge during stress responses (Matsuura *et al.* 2014).

This review explores the advances during the last two decades in research of southern Brazilian *Psychotria* species towards sustainable plant biomass production and elicitation of bioactive alkaloids, focusing on propagation and production of these pharmaceutically relevant target metabolites.

Psychotria L. in southern Brazil

Psychotria (Rubiaceae, Rubioideae, tribe Psychotrieae) is one of the largest genera of flowering plants (Angiosperms) which comprises *ca.* 1,600 species worldwide distributed, being particularly centralized in tropical and pantropical zones (Calixto *et al*. 2016). *Psychotria* spp. are shrubs, arborescent or less frequently herbaceous plants (Dillenburg & Porto 1985; Sobral & Jarenkow 2013) that preferentially occur in shaded understory areas and humid forest soils (Moraes *et al*. 2011; Ferreira-Junior & Vieira 2015). In general, *Psychotria* (*s.l.*) plants feature interpetiolar stipules (distinctive character for Rubiaceae), actinomorphic flowers, and different types of fruits, like schizocarp, capsule, drupe, or berry (Souza & Lorenzi 2012).

As previously described, the genus P*sychotria* L. is well known by the biosynthesis of a number of bioactive alkaloids exhibiting interesting chemical traits and pharmacological effects, besides the ethnobotanical uses reported for several of its species, especially in South America (Farias *et al.* 2012; Calixto *et al.* 2016). However, the taxonomy of this group (at genus level) still remains uncertain, in part due to the morphological high diversity of characters used in description (Moraes *et al*. 2011) and to the similarities found among closely related species between *Psychotria* and *Palicourea*, for example (Both 2005). For this reason, chemotaxonomic (Lopes *et al*. 2004; Henriques *et al*. 2004) and morphological (Moraes *et al*. 2011) investigations have been carried out over the years in an attempt to clarify the unsolved *taxa* delimitations and nomenclatural problems in *Psychotrieae* tribe, mainly in *Psychotria* (Tab. S1 in supplementary material).

A total of 243 native *Psychotria* spp. (including five subspecies and two varieties) occur in Brazil; among those, 137 species are classified as endemic to this country (Reflora 2018). From the *Psychotria* spp. listed in the Brazilian Flora, six species widely distributed in southern Brazil (Fig. 1) have been analyzed for their chemical properties, as follows: ***Psychotria brachyceras* Müll. Arg.**: basionym of *Uragoga brachyceras* (Müll. Arg.) Kuntze; ***P. carthagenensis* Jacq.**: basionym of *Uragoga carthagenensis* (Jacq.) Kuntze; ***P. leiocarpa* Cham. & Schltdl.**: basionym of *Uragoga leiocarpa* (Cham. & Schltdl.) Kuntze; ***P. myriantha* Müll. Arg.** (*Palicourea mamillaris* (Müll. Arg.) C.M. Taylor is the currently accepted name): basionym of *Psychotria mamillaris* Müll. Arg. and *Uragoga mamillaris* (Müll. Arg.) Kuntze; ***P. suterella* Müll. Arg.**: basionym of *Uragoga suterella* (Müll. Arg.) Kuntze; and ***P. umbellata* Vell.** (*Psychotria brachypoda* (Müll. Arg.) Britton is the currently accepted name): basionym of *Uragoga umbellata* (Müll. Arg.) Kuntze (Tropicos 2018). Bioactive alkaloids have been found in all of the above cited species in southern Brazil, except *P. carthagenensis* Jacq. (Porto *et al.* 2009).

Alkaloid characterization and potential biological activities

Alkaloids are known since the early 1800's, when morphine was first described due to extensive human use of opium. Indeed, plant alkaloids are relevant natural products because of their pharmacological activities. Codeine, a very popular antitussive, was isolated from *Papaver somniferum* (Papaveraceae), the same species from which morphine was discovered. Vincristine and vinblastine are remarkable molecules applied in cancer treatments found in *Catharanthus roseus* (Apocynaceae). Also, quinine, obtained from *Cinchona* sp. (Rubiaceae), was used as a model for production of chloroquine, an antimalarial drug (Kutchan *et al.* 2015).

Alkaloids can be characterized by the presence of nitrogen heterocyclic rings. Usually, alkaloid biosynthesis is derived from amino acids (Kutchan *et al.* 2015). *Psychotria*

spp. produce a great diversity of specialized metabolites, including the peculiar monoterpene indole alkaloids that retain glucoside residues (Fig. 2). Monoterpene indole alkaloids (MIA) are derived from the condensation of tryptamine to a terpene moiety, usually secologanin (derived from the 2-C-methyl-D-erythritol 4-phosphate or MEP pathway), by vacuolar strictosidine synthase (STR) (O'Connor & Maresh 2006). In general, evidence suggests MIA alkaloid biosynthetic pathways are complex and divided among different organelles, cell types and tissues (Pan *et al.* 2016). Therefore, given the difficulty in reproducing these tissue and cell differentiation-related microenvironment conditions and fine biochemical regulation in simpler undifferentiated cell culture or microbial systems, biotechnological approaches targeting higher alkaloid content in whole plants are still a key focus for the pharmaceutical industry.

Brachycerine

Psychotria brachyceras synthetizes brachycerine (Fig. 2), an unusual MIA that has a terpene moiety most likely derived from epiloganin (Kerber *et al.* 2001). In field-grown plants, brachycerine content was higher in inflorescences (0.3 % dry weight (DW)), followed by fully expanded leaves/branches (0.2 % DW), young leaves (0.12 % DW), and fruits (0.04 % DW). The alkaloid was not detected in root cells, and brachycerine accumulation occurs in rootless tip cuttings (Kerber *et al.* 2001; Gregianini *et al.* 2003; 2004).

Brachycerine concentration in leaves may vary seasonally, reaching higher levels during spring. Also, evidence suggests that differences can exist in basal content of brachycerine among individuals. It was possible to identify high alkaloid accumulating individuals (*e.g.* 0.74 ±0.25 % DW) and low accumulating ones (*e.g.* 0.25 ±0.02 % DW), with differences standing independently of the season (Gregianini *et al.* 2004).

Brachycerine accumulation was promoted by wounding in a non-systemic fashion (Gregianini *et al.* 2004). Hence, the alkaloid was tested for deterrent activity in two generalist models (*Spodoptera frugiperda* and *Helix aspersa*). *Psychotria carthagenensis* was also tested because it is a co-occurring species that lacks MIA. Leaf extract of *P. carthagenensis* showed some deterrence in snail model, but brachycerine could not deter either of the animals. Further analysis suggested that soluble tannin accumulation in *P. carthagenensis* (2.7 mg/g DW) could partly explain the deterrent activity (Porto *et al.* 2014). The subsequent isolation and characterization of cyclotides (toxic cyclic peptides) in leaves of *P. brachyceras* and *P. leiocarpa* shed some light on anti-herbivore defense mechanisms in these species (Matsuura *et al.* 2016b).

Because some of the alkaloid-accumulating species occur as homogeneous groups in the forest understory, the possibility of phytotoxic effects of the alkaloids was examined on lettuce (*Lactuca sativa* (Asteraceae)) as target plant. However, no inhibitory effects of brachycerine were observed on germination percentage, kinetics or early growth of seedlings (Fig. S1 in supplementary material).

Brachycerine was strongly induced by heat in leaf disks of *P. brachyceras*. When applied to the leaf disks of heat sensitive species *Brugmansia suaveolens* (Solanaceae) and

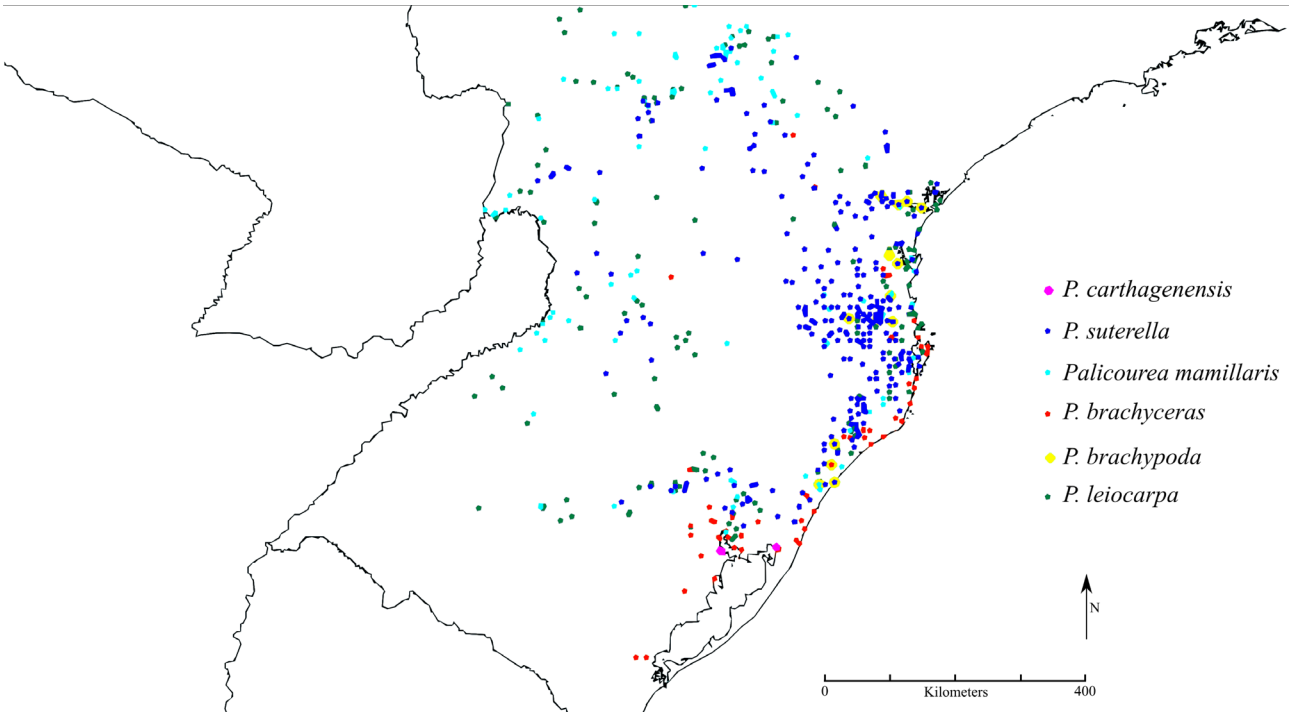

Figure 1. Distribution map of southern Brazilian *Psychotria* species.

Brassica oleracea var. *acephala* (Brassicaceae), brachycerine prevented chlorophyll loss upon heat shock at 50 °C for 6 h. Brachycerine concentration tested was similar to that found in the leaves of *P. brachyceras* field-grown individuals (Magedans *et al.* 2017).

A growth inhibition assay, using mutant strains of *Saccharomyces cerevisiae* deficient in antioxidant enzymes was done to test brachycerine antioxidant activity against hydrogen peroxide (H_2O_2) and paraquat. Brachycerine was effective against both oxidative stressors, but it proved more efficient against paraquat, a superoxide generator. *P. brachyceras* leaf extract also showed pronounced antioxidant activity against oxidation produced by both H_2O_2 and paraquat (Nascimento *et al.* 2007).

Brachycerine mitigation activity towards hydroxyl radical ($OH^{\cdot}$) worked in a dose-dependent manner, as demonstrated through the hypoxanthine/xanthine oxidase assay (Nascimento *et al.* 2007). Also, brachycerine was capable of protecting rubrene from photoxidation by auto-generated singlet oxygen upon exposure to visible or UV-C light. These results indicate the alkaloid has quenching activity against singlet oxygen. This could be related to brachycerine chemical structure, which includes a secondary amine, hydroxyl group and a glucose residue, all of which may act as quenching sites. Also, brachycerine absorbs UV radiation, potentially acting as a filter (Fig. 2); indeed, its accumulation is strongly promoted by acute UV exposure of leaves (Gregianini *et al.* 2003).

The lack of toxic effects of brachycerine on lepidoptera, gastropoda, and yeast has led to the hypothesis that brachycerine and other major *Psychotria* spp. MIAs could modulate oxidative damage caused by herbivory and other environmental stresses, whereas cyclotides would play a role in deterrence (Matsuura *et al.* 2016b).

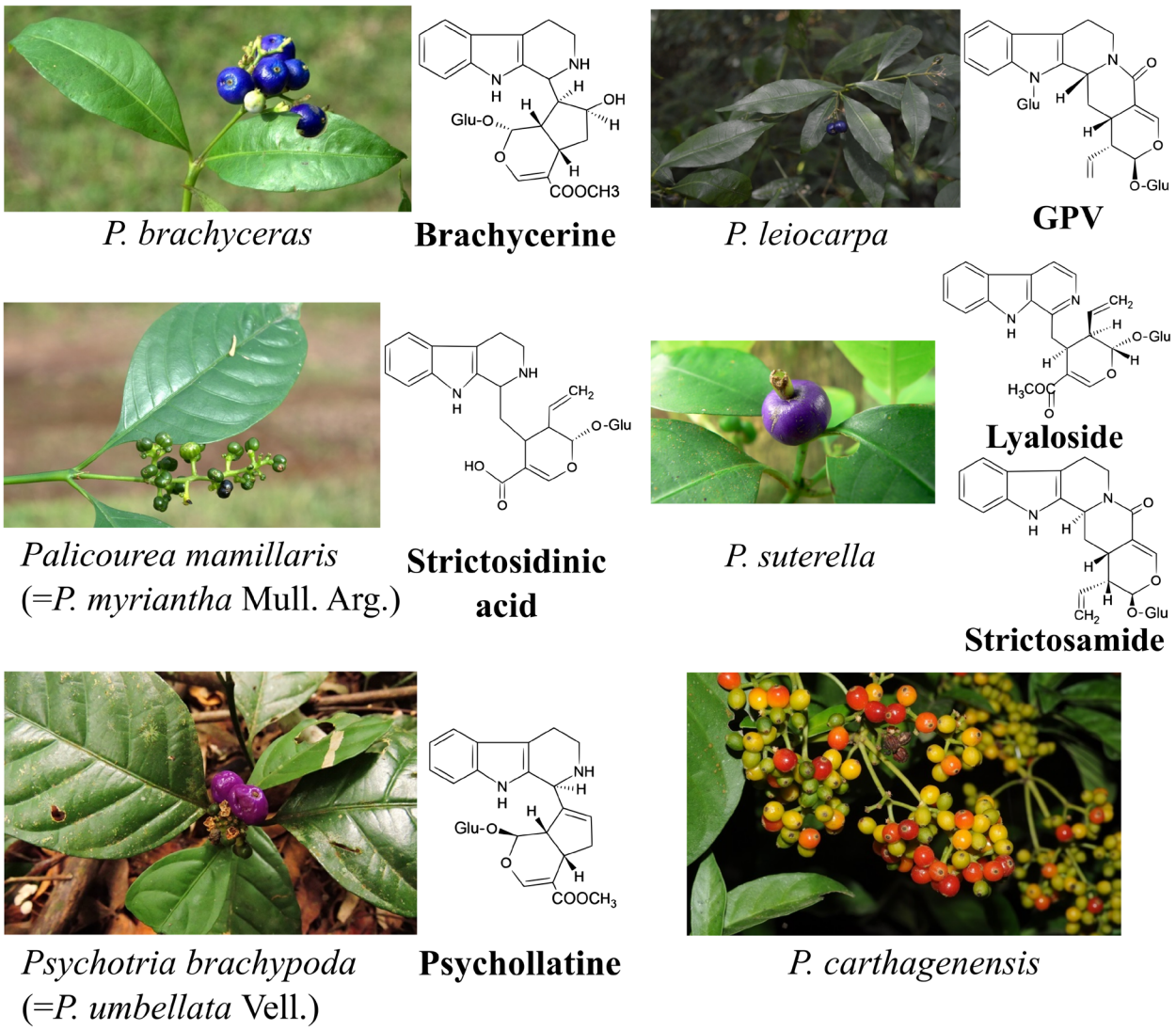

Figure 2. Monoterpene indole alkaloids from southern Brazilian *Psychotria* species. Plant images were taken from Flora Digital, available at http://www.ufrgs.br/fitoecologia/florars/. Credits: Sérgio Bordignon (*P. brachyceras, P. carthagenensis, P. mamillaris*), Ronaldo Jr. (*P. brachypoda*), Denis Fedrizzi (*P. leiocarpa*), Martin Molz (*P. suterella*).

Psychollatine

Psychollatine was first described in *Psychotria brachypoda* (= *Psychotria umbellata*) under the name "umbellatine" (Both *et al.* 2002b). Psychollatine structural validation through NMR data analysis was carried out (Kerber *et al.* 2008) (Fig. 2). Psychollatine is presumably synthetized by the condensation of tryptamine with a geniposide derivative, an alternative MIA biosynthetic pathway. Later, three new MIAs that possibly derive from psychollatine metabolism were described (Kerber *et al.* 2014).

Considering vegetative organs, leaves are the main source of psychollatine (3.7 % DW), followed by stems (1.01 % DW). The alkaloid was also present in several reproductive structures, such as immature inflorescences (4.52 % DW), fruit pulp (2.85 % DW), and seeds (0.21 % DW) (Paranhos *et al.* 2009). The regulation during development suggests a potential defense role for psychollatine (Matsuura *et al.* 2013); however, tests using *Spodoptera frugiperda* with up to 9 mM of the alkaloid added to its food showed no deterrence or toxicity signs, akin to what was recorded for brachycerine. Although psychollatine leaf content did not vary seasonally, it was possible to identify high and low producing field-grown individuals. Psychollatine content is very stable in leaves after harvest, as long as temperatures are kept moderate (Paranhos *et al.* 2009).

Mild analgesic activity was described for psychollatine (200 mg/kg) in hot plate and tail flick tests (Both *et al.* 2002b). Also, psychollatine was considered a 5HT2A/C serotonin modulator in mice investigation models of depression, anxiety and memory (Both *et al.* 2005). For example, in forced swimming test for antidepressant effect, psychollatine (3 and 7.5 mg/kg), was comparable to imipramine (15 mg/kg) and fluoxetine (20 mg/kg) (Both *et al.* 2005). Also, it was suggested that N-methyl-D-aspartate (NMDA) glutamate receptors partially explain psychollatine mechanism of action (Both *et al.* 2006).

Psychollatine antioxidant activity was evaluated *in vitro*. In yeast growth inhibition assay, the alkaloid was more efficient against paraquat, whereas the leaf extract was more effective mitigating H_2O_2-mediated oxidative stress. It was not observed a dose-dependent response for these reactive oxygen species, unlike what was detected for in vitro assays against hydroxyl radical (Fragoso *et al.* 2008).

When compared to other *Psychotria* species, *P. brachypoda* (= *P. umbellata*) aqueous leaf extracts had the strongest inhibitory effect on germination of *Lactuca sativa* (Corrêa *et al.* 2008). Nonetheless, as shown for brachycerine, these phytotoxic effects were not related to the alkaloid (Fig. S1 in supplementary material).

Strictosidinic acid

The major alkaloid from *Palicourea mamillaris* (= *Psychotria myriantha*) is strictosidinic acid (Fig. 2). The ethanolic leaf extract also yielded another compound, named myrianthosine after purification and structural elucidation of alkaloid fractions (Simões-Pires *et al.* 2006).

Strictosidinic acid could prevent *in vitro* polymorphonuclear leukocytes chemotaxis (Simões-Pires *et al.* 2006), which suggests anti-inflammatory activity. Other *Psychotria* have been used in traditional medicine as anti-inflammatory agents (Alonso-Castro *et al.* 2011).

When isolated from *Hunteria zeylanica* (Apocynaceae), strictosidinic acid showed analgesic and antipyretic activity in mice models (Reanmongkol *et al.* 2000). Also, the crude leaf extracts of *P. myriantha* showed analgesic potential (Both *et al.* 2002a). These results motivated further investigation on the alkaloid action in the central nervous system.

Intraperitoneal injection of strictosidinic acid (10 mg/kg) reduced monoamine levels in rats, revealing a possible relevant effect in the central nervous system (Farias *et al.* 2010). Intra-hippocampal injection (20 µg/µl) of strictosidinic acid reduced serotonin levels in Wistar rats; intraperitoneal application (10 mg/kg) could also reduce serotonin and DOPAC (3,4-dihydroxyphenyl acetic acid) levels. These results suggest a possible role of the alkaloid in the dopaminergic transmission, further indicated by its inhibition on monoamine oxidase activity (Farias *et al.* 2012).

Lyaloside and strictosamide

Psychotria suterella main MIAs are lyaloside, strictosamide (Fig. 2), and naucletine. These alkaloids were isolated from leaf extracts, but were not found in root cultures or callus cultures. Analgesic effects of leaf extract (300 mg/kg) or lyaloside (30 mg/kg) were not significant in tail flick test and higher doses caused animal death (Santos *et al.* 2001).

Lyaloside, strictosamide (Fig. 2), and leaf alkaloid fractions of *P. suterella* were used in monoamine oxidase (MAO-A/B) inhibition experiments. Fractions were more effective than lyaloside and strictosamide in depleting MAO activity. A trend to preferentially inhibit the isoform MAO-A could be observed. MAO-A inhibitors have potential pharmacological use in depression treatment (Passos *et al.* 2013).

GPV

Psychotria leiocarpa major MIA is *N*,β-D-glucopyranosyl vincosamide (GPV) (Fig. 2) (Henriques *et al.* 2004). Leaves of adult field-grown trees can accumulate up to 2.5 % of their total dry weight as GPV. GPV biosynthesis seems to be developmentally regulated. For instance, young leaves accumulated relatively more GPV than fully expanded ones. Also, during reproductive stages, there is a commitment with GPV accumulation at early stages, as floral buds and open flowers showed highest levels of the alkaloid (Matsuura *et al.* 2016a).

There is evidence suggesting light is a necessary condition for GPV biosynthesis and accumulation at high levels in seedlings. Dark grown *P. leiocarpa* plantlets did not show significant increase in GPV content after 14 days of light exposure. On the other hand, light-grown individuals, when transferred to dark, had considerable decrease in GPV concentration (Matsuura *et al.* 2016a).

Aqueous leaf extracts (4% w/v) of *P. leiocarpa* inhibited germination rate and initial growth of diaspores cultivated in Petri dishes (*L. sativa*) and in soil (*Mimosa bimucronata* (Fabaceae)). However, purified GPV was not able to induce those effects. Polar phenolic compounds or iridoids were most likely responsible for the phytotoxic effects, based on activity-guided solubility tests (Corrêa *et al.* 2008). Oil components were also evaluated in *P. leiocarpa* leaves. Mainly sesquiterpenes bearing germacrane and cadinane skeletons were identified (Andrade *et al.* 2010).

GPV deterrence activity was tested in two generalist models (*S. frugiperda* and *H. aspersa*) and in a specialist herbivore model (*Heliconius erato* on *Passiflora suberosa*). GPV treatments simulated alkaloid concentrations found in natural conditions, but deterrence effect was not observed in any case (Matsuura & Fett-Neto 2013). However, as observed for *P. brachyceras*, *P. leiocarpa* cyclotides could explain overall herbivore deterrence of the species observed in field conditions (Matsuura *et al.* 2016b).

GPV has significant *in vitro* quenching activity towards singlet oxygen, superoxide anions, and hydroxyl radical. Hydroxyl radical mitigation by GPV was further corroborated with an *in situ* experiment localizing hydrogen peroxide by reaction with diamino benzidine. When directly compared to brachycerine and psychollatine, GPV appeared to be the most efficient antioxidant (Matsuura *et al.* 2016a).

GPV application on leaf disks of UV-B sensitive *P. carthagenensis* and *Phaseolus vulgaris* prevented chlorophyll loss after 48 h up and to 96 h of acute UV-B exposure. These species do not have MIAs. Therefore, evidence suggests GPV antioxidant activity could mitigate severe UV-induced oxidative stress, preventing chlorophyll loss (Matsuura *et al.* 2016a).

Strategies for clonal and sexual propagation of *Psychotria* spp.

The successful propagation of plant genotypes with relevant pharmaceutical interest is a key step to obtain adequate amounts of useful compounds. This becomes especially relevant when considering that most commercially prospected plant species have a restricted distribution, sometimes only in scarce natural populations. These resources could become limited if extensively exploited. Efficient methods of plant propagation and cell culture are crucial to improve yields of the desired products without threatening natural resources. Hence, different techniques have been established to propagate *Psychotria* species, including rooting of cuttings, seed germination, somatic embryogenesis induction, and development of cell and organ culture protocols (Tab. 1, Fig. 3).

Cuttings

Clonal propagation of *P. brachypoda* (= *P. umbellata*) was performed through the rooting of apical shoots obtained from adult trees, with two to six leaves (Paranhos 2003),

Figure 3. Strategies of plant propagation for *Psychotria* spp. **A.** Cuttings from *P. brachyceras*. **B.** Rhizogenic cultures from *P. suterella* Mull. Arg. **C.** *Psychotria brachyceras* calli.

Table 1. Methods of plant propagation and cell culture for *Psychotria* spp.

Propagation strategy	Species with established protocols	References
Cuttings	*P. brachypoda* and *P. brachyceras*	Kerber *et al.* 2001; Paranhos 2003
Seeds	*P. leiocarpa* and *P. brachyceras*	Rosa & Ferreira 2001; Paranhos 2003; Henriques *et al.* 2004
Somatic embryogenesis from rhizogenic callus	*P. brachypoda*	Paranhos *et al.* 2005
Callus cultures	*P. brachypoda*, *P. suterella* and *P. brachyceras*	Santos *et al.* 2001; Gregianini *et al.* 2003; Paranhos *et al.* 2005
Plant cell cultures	*P. brachyceras*	Limberger *et al.* 2007
Root cultures	*P. suterella*	Santos *et al.* 2001

as previously described for *P. brachyceras* (Kerber *et al*. 2001). Two concentrations of the auxins indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) (0 and 10 mg.l^{-1}) were tested in a hydroponic system containing water or MS (Murashige & Skoog 1962) nutritive solution at 0.1x and 0.2x strength (Paranhos 2003). Cuttings were kept in the media with phytohormones for one week and then transferred to media devoid of auxins thereafter. The cuttings were maintained in a growth chamber at 28±2 ºC, with photoperiod of 16 h of light and ~73 µmol.m-2.s-1 of photosynthetically active radiation (PAR) to monitor root development. Better rooting performance was observed using IBA in 0.1x MS, with higher rooting percentage and cutting survival. IAA did not cause differences in rooting and survival percentage in relation to control. The amounts of psychollatine accumulated in the stems and roots of the cuttings were higher than in plants grown in the field, but no difference was observed in leaves, which accumulate the highest levels of this metabolite in both field and growth chamber conditions. Cuttings have been used for elicitation and alkaloid determination assays to investigate the environmental control of their accumulation, as well as a source of explants for tissue culture studies in *P. brachypoda* (= *P. umbellata*) and *P. brachyceras* (Tab. 1, Fig. 3) (Gregianini *et al*. 2003; 2004; Paranhos *et al*. 2005; 2009; Limberger *et al*. 2007; Nascimento *et al*. 2013a).

Seeds

The medium with the best results obtained for *P. brachypoda* (= *P. umbellata*) was used in an attempt to obtain rooted cuttings of *P. leiocarpa*, but the rooting and survival percentages were low for this species (Paranhos 2003). Thus, *in vitro* seedlings were obtained from seeds to examine the accumulation of GPV (Paranhos 2003; Henriques *et al*. 2004). Seeds were surface-sterilized and germinated in solid MS 0.1x culture media with (15 g.l-1) or without sucrose under a photoperiod of 16 h of light (~40 µmol.m-2.s-1 of PAR) or in continuous dark at 28±2 ºC (Paranhos 2003). Although presence or absence of sucrose did not yield any difference, better germination percentages were observed in presence of light (around 40 %) in comparison with continuous dark (around 10 %). Also, a period of 10 ºC for one day or one week prior to sowing, improved germination values in relation to the seeds that were kept at 28 ºC (Paranhos 2003). In another assay, the best germination performances were observed at 25 ºC, ranging from 62 to 66 %, in the presence and absence of light, respectively (Rosa & Ferreira 2001), suggesting that seed batch can affect the success rate of germination. Germination in this species can take several weeks (Rosa & Ferreira 2001; Henriques *et al*. 2004). Seedlings of *P. leiocarpa* were able to accumulate GPV, although at lower levels than adult plants (Henriques *et al*. 2004), which might be related to their stage of development. Plants grown from seeds have been used for the clonal propagation of *P. leiocarpa* and *P. brachyceras* (Henriques *et al*. 2004; Gregianini *et al*. 2004; Matsuura *et al*. 2016a).

Somatic embryogenesis

Internodal stem segments from rooted tip cuttings of *P. brachypoda* (= *P. umbellata*) were used to establish a protocol for plant regeneration from totipotent callus (Paranhos *et al*. 2005). Rhizogenic calluses were obtained from explants cultured in MS media with the auxin naphthalene acetic acid (NAA) or with NAA plus the cytokinin kinetin (KIN). Subsequently, different concentrations of KIN and sucrose were tested in the regeneration media under light or darkness aiming to obtain plants from callus slices through somatic embryogenesis. The best results were achieved when the segments were cultured in light, with MS media containing 0.25 mg. l-1 of KIN and 1.5 % of sucrose, a combination that reached 60 % of plant regeneration. Although psychollatine was not produced by calli, the regenerated plants were able to yield amounts of this indole alkaloid comparable to those of the plants growing in the forest. This methodology seems promising to a sustainable production of psychollatine, since about 150 plants were obtained from each callus slice in less than a year. The lack of alkaloids in the calli was also previously observed for *P. brachyceras*, which did not produce brachycerine in dark-grown callus cultures and only traces of this alkaloid were observed by HPLC in green calli under ultraviolet radiation exposure (Gregianini *et al*. 2003). In *Psychotria suterella*, the alkaloids were not detected either in seedling-derived root cultures or leaf-derived callus cultures (Santos *et al*. 2001). Taken together, evidence suggests that the accumulation of these alkaloids depends on differentiated shoots.

Plant cell cultures

Cell suspension cultures established from calli of *P. brachyceras*, which do not accumulate brachycerine, were used for biotransformation assays (Limberger *et al*. 2007). The callus tissues were induced from stem segments of cuttings (Fig. 3). The cell suspension cultures started from 30 g of callus tissue in media with the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) and sucrose. After one week, substrates were added to the media with cells to observe biotransformation of (1*S*,5*R*)-(-)-alpha-pinene and (1*R*,5*S*)-(+)-alpha-pinene into (-)- and (+)- verbenone. The protocol was successful and the cells of *P. brachyceras* were able to generate mainly the food flavor and pine bark beetle dispersant (-)-verbenone. Although relatively little is known about biotransformation reactions using *Psychotria* species, use of cell suspension cultures as biocatalysts is a potential strategy to produce target compounds in a fast and continuous way for commercial supply (for review, see Matsuura *et al*. 2018).

Elicitation techniques for increasing alkaloids content *in vivo*

Plant metabolism evolved to cope with daily and seasonal variation of abiotic and biotic factors. External stimuli induce molecular changes in a complex and elaborate network that starts at perceiving stimuli, transmitting signals through cells, followed by activating regulatory components and biosynthetic pathways. These responses are key properties that afford overall fitness increase in plants facing different environmental conditions throughout their lifetime (Kutchan *et al.* 2015).

There are a number of propagation systems to produce high quantities of good quality plant biomass towards extraction of bioactive alkaloids. In addition, it is possible to increase alkaloid content in *Psychotria* species by applying abiotic stress or signals of biotic interactions for a relative short period. Reactive oxygen species may act as signaling molecules triggering alkaloid biosynthesis. Alkaloids, on their turn, could feedback regulate these stress-derived damaging reactive species thanks to their broad antioxidant activity (Matsuura *et al.* 2014). Plant hormones could mediate MIA biosynthesis as well, such as abscisic acid and jasmonate. Herein, we will present different elicitation techniques for increasing *Psychotria* alkaloids *in vivo*.

When exposed to 16 h of UV-C per day, *P. brachyceras* cuttings produced 10 times more brachycerine (in a six days experiment), whereas 4 h of daily exposure yielded only a two-fold increase of the alkaloid. Interestingly, leaves that fell during test had similar brachycerine content as those attached. This indicates *de novo* brachycerine synthesis in leaves is preferable over metabolite translocation under UV stress (Gregianini *et al*. 2003). On the other hand, UV treatments could not increase alkaloid content in leaf disks of *P. brachypoda* (= *P. umbellata*) and *P. leiocarpa*, in spite of the fact that both species showed relatively high tolerance to this kind of stress (Paranhos *et al.* 2009; Matsuura & Fett-Neto 2013). In these cases, basal alkaloid levels may be sufficient to protect plant tissues.

Psychotria brachyceras gene expression under UV stress was analyzed. Differentially expressed genes in leaves under acute UV-B stress for 24h were selected by suppression subtractive hybridization assay. *TRYPTOPHAN DECARBOXILASE* had a 5-fold expression increase and an *UDP-GLUCOSE GLUCOSYL TRANSFERASE* showed a 4-fold increase in expression, which was also observed for some genes related to jasmonate and ethylene biosynthesis (Nascimento *et al.* 2013b). Hence, at least part of the increased alkaloid accumulation in response to UV has putative regulation at transcript level. In fact, *P. brachyceras* proved to be highly tolerant to acute UV exposure (DD Porto & AG Fett-Neto, unpubl. res.).

GPV accumulation in seedlings was promoted by light and partly inhibited by supplying exogenous sucrose, indicating dependence on photoautotrophic shoots for accumulation, which has been tentatively attributed to low terpene moiety production by the plastid MEP pathway under limited photoautotrophic conditions (Henriques *et al.* 2004). Light quality affected GPV accumulation in *P. leiocarpa*. Far-red and blue light enrichments stimulated GPV accumulation in shoots of seedlings after 10 days of experiment (Matsuura *et al.* 2016a).

Mechanical damage was also tested for alkaloid elicitation. Leaves from tip cuttings of *P. brachyceras*, *P. brachypoda* (= *P. umbellata*) and *P. leiocarpa* were subjected to mechanical damage. Brachycerine content doubled after 24 h and returned to basal levels after 48 h. Brachycerine elicitation was essentially restricted to the wound site (Porto *et al.* 2014). Psychollatine and GPV content did not vary after mechanical damage, showing a phytoanticipin-like accumulation profile, *i.e.* mostly constitutive (Paranhos *et al.* 2009; Matsuura & Fett-Neto 2013).

Leaf disks have been extensively used to test the effect of abiotic stress and signaling molecules on alkaloid content of *Psychotria*. This method is useful to avoid longer-term cutting cultivation and to favor homogenization of genetic effects within experiments by randomly mixing the disks of several different trees. Briefly, tip cuttings from several individuals of one or more populations are acclimated for one week in 0.1x (v/v) MS salts solution (pH 5.8) in a growth room. Then, leaves are sterilized with sodium hypochlorite for disk preparation using a sharp cork borer (1cm of diameter). Leaf disks are randomly mixed and placed in Petri dishes containing filter paper moistened in the same MS media described above (Magedans *et al.* 2017). Disks are viable and do not lose significant chlorophyll for at least four to five days.

Psychotria brachyceras leaves were treated with 40 μM of jasmonate, which increased brachycerine content by 2.7-fold after 6 days of treatment. A higher dose of jasmonate (400 μM) caused brachycerine content induction of 3.3-fold, 4 days after jasmonate application (Gregianini *et al.* 2004), suggesting a phytoalexin-like accumulation profile, *i.e.* increased upon stimulus. Consistent with its phytoanticipin-like production profile, GPV could not be induced by jasmonate (Matsuura & Fett-Neto 2013).

Salicylic acid application did not affect brachycerine and psychollatine contents (Nascimento *et al.* 2013a; Paranhos *et al.* 2009), although mechanical wounding could increase brachycerine content in tip cuttings. Auxin exposure decreased psychollatine content in leaf disks at multiple exposure times tested (Paranhos *et al.* 2009). Overall, psychollatine has a phytoanticipin-like accumulation profile, similar to that described for GPV.

Osmotic agents were tested as elicitors in *P. brachyceras* leaf disks. Sorbitol (0.1 M), sodium chloride (0.005 M) and polyethylene glycol (PEG – 0.05 M) strongly promoted brachycerine accumulation. Abscisic acid (1 mg/L), a plant hormone implicated in drought stress, could induce a 3.5-fold increase in brachycerine content at the third day of experiment (Nascimento *et al.* 2013a). These data support a role for drought in stimulating brachycerine accumulation, corroborating data on seasonal content variation of field-grown trees. Heavy metal exposure could also induce alkaloid content in *P. brachyceras* leaf disks. Aluminum chloride (30 μM) and silver nitrate (2.3 μM) induced brachycerine content up to 3-fold, suggesting that metal and/or drought-triggered oxidative stress plays a role in alkaloid biosynthesis. Indeed, PEG (0.05 M) treatment significantly induced ascorbate peroxidase (APX) activity, at 6h and 12h after the onset of the experiment. However, this was not observed for superoxide dismutase (SOD) activity (Nascimento *et al.* 2013a), suggesting a SOD-like role of brachycerine, particularly given its powerful capacity to quench superoxide ions. Hydrogen peroxide application on *P. brachypoda* (= *P. umbellata*) leaf disks did not affect psychollatine content (Paranhos *et al.* 2009), in line with its constitutive accumulation profile.

Heat stress is also a tool for alkaloid elicitation in *P. brachyceras*. Leaf disks were exposed to temperature increase from 25 °C to 40 °C in two experiments. In the first one, temperature was changed abruptly, and leaf disks were kept for 3 days at 40 °C. In the second test, temperature was increased stepwise, *i.e.* 5 °C increase per day in a one-week experiment. Brachycerine content increased up to 2-fold in both experiments. The threshold temperature to initiate brachycerine accumulation was 40 °C. Interestingly, *TRYPTOPHAN DECARBOXYLASE* expression decreased in leaf disks exposed to acute change of temperature, at 12 h and 24 h, suggesting that control of brachycerine accumulation by heat is mostly post transcriptional. In agreement with this indication, leaf disks exposed to heat treatment had higher content of tryptamine and TDC activity (Magedans *et al.* 2017).

Conclusion and perspectives

Psychotria spp. of southern Brazil represent a valuable reservoir of alkaloids with diverse bioactivities of commercial interest. Among the main general features of MIAs in these Brazilian *Psychotria* species are: a) presence of glucose residues; b) accumulation at relatively high levels in shoots; c) absence in roots, root cultures, callus and cell cultures; d) lack of overt toxicity to herbivores or other plants; e) high antioxidant capacity against reactive oxygen species, f) capacity to improve responses to stress (*e.g.* heat and UV) *in vivo* when applied to sensitive plant species and to yeast mutants deleted in enzymatic antioxidant defense systems.

Propagation strategies for these species are in place and can provide catalytically active biomass for alkaloid extraction. Both clonal and seed-based propagation protocols may be used, ensuring rapid germplasm improvement and uniformity, as well as preservation of genetic bases for rescuing needed characteristics (*e.g.* pathogen resistance) if required.

Perspectives for research include a deeper examination of putative alkaloid function *in planta*, which has been proving useful for identification of new bioactivities. Probably, there are several untapped alkaloid sources, mechanisms of accumulation, and new bioactivities to be explored in southern Brazilian *Psychotria*, all of which must be addressed in future studies. Undoubtedly, the understory trees of the forest hide many undiscovered chemical treasures just waiting to surface.

Acknowledgements

The authors would like to acknowledge the financial support from the Coordination of Improvement of Higher Level Personnel (CAPES-Brazil-finance code 001) and the National Council for Scientific and Technological Development (CNPq-Brazil, grant 303560/2017-7). In 2019, as the 50th birthday of the Graduate Program in Botany of the Federal University of Rio Grande do Sul is celebrated, we would like to express our gratitude to the dedicated professionals and students that made the Program´s history a reality.

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