Article

In Vitro Cultivated *Uncaria tomentosa* and *Uncaria guianensis* with Determination of the Pentacyclic Oxindole Alkaloid Contents and Profiles

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Uncaria tomentosa (Willd.) DC. e *Uncaria guianensis* (Aubl.) Gmel., conhecidas comercialmente como unha-de-gato, são trepadeiras lenhosas nativas das florestas tropicais amazônica e central-americana. Essas espécies de Rubiaceae enfrentam ultimamente um eminente risco de extinção devido tanto ao extrativismo indiscriminado quanto ao desflorestamento de seus habitats naturais. Esse trabalho descreve métodos de cultivo *in vitro* para as duas espécies, analisando o perfil e o teor dos alcalóides oxindólicos pentacíclicos marcadores por CLAE no material vegetal obtido. Os resultados mostraram que os métodos desenvolvidos foram capazes de produzir plantas com perfil e teor dos alcalóides alvos similares à plantas nativas e cultivadas *in vivo*, com a vantagem adicional de produção em menor tempo de mudas próprias para plantio no campo. Os dados evidenciaram que o cultivo *in vitro* pode ser uma ferramenta viável para a produção das duas espécies com impacto na sua comercialização e na preservação das florestas.

Uncaria tomentosa (Willd.) DC. and *Uncaria guianensis* (Aubl.) Gmel., commercially known as cat's claw, are large woody vines native of the Amazon and Central America rainforests. These Rubiaceae species face nowadays an imminent risk of extinction due to indiscriminate harvesting in the wild as well as to increasing deforestation of their natural habitat. This work describes *in vitro* cultivation methods for both species with determination, by HPLC, of the pentacyclic oxindole alkaloid contents and profiles of the plant material. The results have proved that the methods we developed were able to produce plants with alkaloid profiles and contents similar to the wild and *in vivo* cultivated species, and with the additional advantage of producing suitable young plants in a shorter period of time. The data showed that the i*n vitro* technique can be a feasible tool for the growth of the species, and may thus be important for their commercialization and for their conservation as a forest resource.

Keywords: *Uncaria tomentosa*, *Uncaria guianensis*, cat's claw, micropropagation, oxindole alkaloids

Introduction

The species *Uncaria tomentosa* (Willd.) DC. and *Uncaria guianensis* (Aubl.) Gmel. (Rubiaceae) known

as cat's claw are large woody vines native of the Amazon rainforest and other tropical areas of South and Central America.[1] They have been used in traditional medicine practically in largely similar ways to treat several ills such as gastritis, gastric ulcers, cancer, arthritis, asthma and inflammatory conditions.[2-4] The species *U. guianensis*

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showed experimentally anti-inflammatory and anti-oxidant activities and *U. tomentosa* is noted for its immunostimulant activity, as well as cytotoxic, anti-inflammatory and anti-oxidant action.[5-7] The bioactivities of the species have made them valuable plant materials and led to them being commercialized *in natura* or as phytopharmaceutical derivatives.[8] Both species contain, in different proportions, tetra- and pentacyclic oxindole alkaloids, indole alkaloids, triterpenoid glycosides, sterols and flavonoids.[6] *In vitro* and *in vivo* assays have demonstrated that the pentacyclic oxindole alkaloids (POA) were the main source of the immunostimulant and cytotoxic activity of *U. tomentosa*[6,9] and led to them being considered biochemical markers of this species and been used to assure the quality of the plant material and commercial herbal medicines.[10-13] The concentration of these alkaloids varies in the different parts of the plants as well as seasonally and they are usually more abundant in *U. tomentosa* than in *U. guianensis*.[14,15] The species face nowadays an imminent risk of extinction due to indiscriminate harvesting in the wild as well as to increasing deforestation of their natural habitat.[3,16] The medicinally used parts are root bark, stem bark and leaves harvested from specimens with an age of *ca.* 8-10 years.[3,17] In order to minimize the impact of the extractivism and look for sustainable use of the species some cultivation initiatives have been made from seeds and/or from root or stem stocks and also by developing ideal harvesting techniques that allow a quicker regrowth.[4,18-20] Still poorly explored, cell culture methods could become a useful tool for propagation of the species as well as for the production of the bioactive oxindole alkaloids. Three studies have reported the development of cell suspension culture methods from leaf explants of *U. tomentosa* aimed at the production of oxindole alkaloids[17,21,22] and another experiment has described cell culture conditions to study the biosynthetic pathways of sterols and triterpenes in *U. tomentosa*.[23]
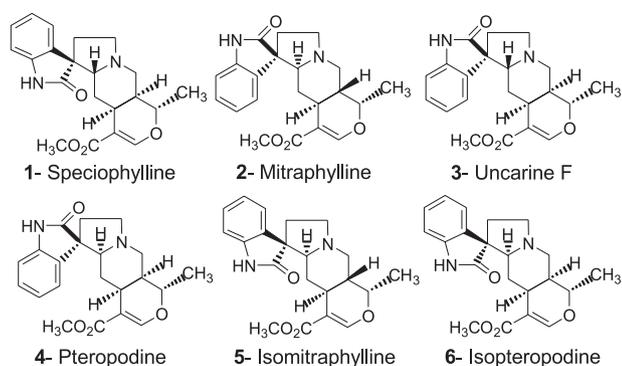
Figure 1. Structures of the pentacyclic oxindole alkaloids regarded as biochemical markers of *Uncaria tomentosa* and *Uncaria guianensis*.

The present investigation describes the POA **1-6** (Figure 1) profiles and their total content through HPLC in micropropagated *U. tomentosa* and *U. guianensis* species. The study seeks to obtain *in vitro* cultivated *Uncaria* species with similar POA profile and content to the wild species in order to contribute to their commercial use and to their conservation as a forest resource.

Experimental

Plant matrices

The seeds utilized to obtain the plant matrices were obtained from Embrapa Acre, Brazil, collected from wild adult specimens in the Brazilian Amazon rainforest in Boca do Acre, Amazonas (*Uncaria guianensis)* and Feijó, Acre (*Uncaria tomentosa*) in August 2003. Voucher species are deposited at the Herbarium of the Zoobotanic Park of the Universidade Federal do Acre (Brazil) under numbers 15640 and 15645, respectively. The seeds of both species were washed in tap water for 10 min and then disinfected in a laminar flow chamber with a 1.25% aqueous solution of sodium hypochlorite for 15 min. After that the seeds were exhaustively washed with autoclaved distilled water for removal of the excess of the disinfectant solution. The aseptic seeds were then germinated during 60 days in a greenhouse using Plantmax® substrate and then transplanted into 4 kg plastic bags with the same substrate and kept under 50% shade and intermittent sprinkling irrigation. After six months the seedlings could be transferred to the field. Leaves (median part of the shoot and anatomic structures fully developed) from these seed cultivated specimens at 24 months were used for the alkaloid determination.

Calli from in vitro leaf explants

Leaf segments of 1 cm^2 were taken from the plant matrices and were inoculated in flasks with 10 mL of Murashige and Skoog (MS) medium[24] containing 1.5 mg L^{-1} of 4-amino-3,5,6-trichloropicolinic acid (Picloram), inorganic salts, inositol, vitamins, 6 g L^{-1} of agar and 30 g L^{-1} of sucrose. After 60 days of continued cultivation, the calli were successively sub-cultivated four times in the same medium. For phytochemical analyses dry calli (ventilated oven at 45°C) with 30, 60 and 90 days after the fourth sub-cultivation were used.

In vitro plantlets

Aseptic seeds of *U. guianensis* obtained as described above and leaf explants of *U. tomentosa* plant matrix

were used for the *in vitro* inoculation. The embryos of *U. guianensis* and the leaf explants from *U. tomentosa* were inoculated in a laminar flow chamber in 25×150 mm test tubes with, for *U. guianensis*, 10 mL of the complete MS culture medium supplemented with 30 g L^{-1} of sucrose, 6.0 g L^{-1} of agar and pH 5.7 and, for *U. tomentosa*, 10 mL of the WPM medium [24] supplemented with 1.0 mg L^{-1} of 6-benzylaminopurine (BAP) and then incubated at $25 \pm 1°C$ with a 16 h photoperiod (15 μmol $(m^2 s)^{-1}$). After 45 days the plantlets of both species were removed from the test tubes and their leaves were excised and dried in a ventilated oven at 30 °C and then submitted to alkaloid analysis. Leaves from three different plantlets of each species were used for the alkaloid determination.

Acclimatization of the in vitro plantlets

After 45 days the *in vitro* produced plantlets (described above) were transferred to a 72 cell tray with 50 mL $cell^{-1}$ of Plantmax® substrate and after 15 days the seedlings were transplanted to 1 kg plastic pots and kept in a greenhouse at the same conditions described for the production of the plant matrices. For alkaloid determination leaves from three different specimens of both species were collected on the 30th and 45th day of the acclimatization and dried in a ventilated oven at 30 °C.

Extraction and quantification of the alkaloids

A mass of 750 mg of the oven-dried and powdered *in vitro* and *in vivo* cultivated plant material from both species were ultrasonically extracted with HPLC grade MeOH (4×2.5 mL) according to the Ganzera *et al.*[12] optimized method. The extractions were made in triplicate. The extracts were filtered, the solvents evaporated under low pressure at 37 °C and the residues weighed. Solutions *ca.* 5 mg L^{-1} were then made with each one by adding the appropriate amount of MeOH. These solutions were added to the macroporous cationic resin Lewatit SP-112 (Bayer) (1:4 extract-mg/resin-mg) and stirred magnetically for 1 h for the retention of the alkaloids.[25] The supernatants were then carefully separated and the resin washed with MeOH (2×5 mL) followed by stirring with MeOH/NH_4OH 1:1 (3×5 mL) for 5 min. The alkaloid-rich supernatants were filtered and the solvents evaporated under low pressure at 37 °C. The resulting fractions were weighed and quantitatively transferred to volumetric flasks with MeOH to yield solutions with concentrations varying from 300 to 700 μg mL^{-1}. These solutions were filtered through 0.45 μm nylon membranes and injected (10 μL) in triplicate into the HPLC system (500E Waters with automatic injector and

photodiode array detector HP series 1100, connected to a HP Asterix Chemstation) equipped with a reverse-phase C_{18} column (Lichrocart Lichrospher 5 μm, 125×4.6 mm i.d.). Elution was carried out with a mixture of 45% of MeCN/55% of aqueous 30 mmol L^{-1} NH_4OAc solution, pH adjusted at 6.8-7.0, isocratic mode, flow rate of 1.0 mL min^{-1}, at 60 °C and detection at 245 nm.[26] The alkaloids peaks were identified by retention time relative to isopteropodine (**6**) in an authenticated POA reference sample described below. Amounts of each POA in the extracts were quantified by external standardization relative to isopteropodine (**6**). This external calibration curve was prepared from injections in triplicate of isopteropodine MeOH solutions at five different concentrations and it presented suitable linearity ($R^2 = 0.994$) in the range of 3.12 to 25.0 μg mL^{-1}. The detectable minimum was determined as 2.4 μg mL^{-1} in the calibration curve and the standard deviation was estimated as 1.0 μg mL^{-1}.

Reference sample

In order to identify each alkaloid peak in the HPLC chromatograms a reference sample was used. This sample is an enriched fraction that contains the six POA obtained through classical acid-base partition[27] from the EtOH-H_2O 1:1 extract of the stem bark of a wild specimen of *U. tomentosa* collected in Cruzeiro do Sul, Acre, Brazil [donated by Biosapiens Co., Brazil – for voucher data see Miranda *et al.*[19]]. This fraction was submitted to reverse-phase HPLC using UV-detector at 245 nm under the same conditions described by Laus and Keplinger[11] with the identification of the POA signals made by comparison of their retention times with those described[27] and also by HPLC-DAD-MS analysis[28] by monitoring the ion $[M+1]^+$ at *m/z* 369 and the corresponding UV spectra.[26] In order to obtain the pure standard compounds, this fraction was suspended in boiling methyl *tert*-butyl ether to precipitate mitraphylline (**2**) that was further purified by crystallization in the same solvent. After evaporation of the solvent at low pressure at 37 °C, the supernatant was sequentially submitted to silica gel column chromatography using a solvent gradient of hexane-EtOAc-MeOH and reverse-phase HPLC.[11,29] The obtained pure compounds were analyzed by ^1H and ^{13}C NMR in one and two dimensions (Bruker DRX-300 spectrometer operating at 300 MHz and using $CDCl_3$ as solvent and TMS as internal reference), FTIR (Nicolet model Magna-IR 760 spectrometer) and HPLC-DAD-MS (2690 Waters chromatography with photodiode array detector Waters 996 model and quadripole micromass ZMD mass spectrometer with electrospray interface) and their data compared with those available in the literature.[28,30]

The purity of the isolated compounds was determinated by integration of the total ion chromatogram in the HPLC-MS. The compounds were obtained with more than 95% purity. Individual compounds were then injected and the retention times relative to isopteropodine (**6**) were determinated: speciophylline (**1**) 0.39, mitraphylline (**2**) 0.45, uncarine F (**3**) 0.48, pteropodine (**4**) 0.59, isomitraphylline (**5**) 0.62 and isopteropodine (**6**) 1.00.

Results and Discussion

Several methods for characterization and quantification of the POA from *Uncaria tomentosa* and *Uncaria guianensis* have been reported [10-12,28,31]. Because of the complexity of the crude alcoholic extracts of both species which contain a large amount of tannins, the oxindole alkaloids have usually been analyzed with a pre-treatment of those extracts with acidic or basic extraction, acid-base partition or solid phase extraction in order to achieve alkaloid-enriched fractions. The present study reports the obtaining of the alkaloid fractions from the developed cultivars by using a solid phase extraction methodology based on cationic exchange.[25] For analytical purposes and as an alternative to the alkaloid standards, a noncommercial reference sample containing the POA **1-6,** obtained by classical acid-base partition from an ethanol extract of the stem bark of a Brazilian wild specimen of *U. tomentosa*, was chemically authenticated. This authentication involved the isolation of the six POA standard compounds whose structures have been confirmed by FT-IR, NMR techniques and HPLC-DAD-MS. Characterization of the six marker compounds in all analyzed samples was related to their relative retention time to isopteropodine (**6**). Amounts of each POA in the extracts were quantified by external standardization relative to isopteropodine (**6**). The alkaloid profile of the reference sample is shown at Figure 2.
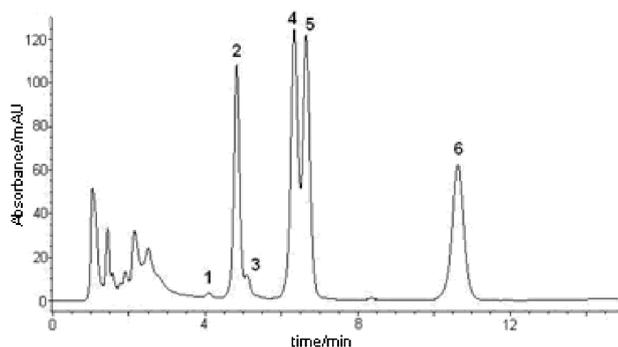
Figure 2. Pentacyclic oxindole alkaloid HPLC profile of the reference sample: **1**: Speciophylline; **2**: Mitraphylline; **3**: Uncarine F; **4**: Pteropodine; **5**: Isomitraphylline; **6**: Isopteropodine.

Plant matrices of both species were produced from seeds. This allowed comparison of the time of development of the seedlings and their alkaloid profiles with plants produced by *in vitro* propagation. These matrices were also used to take leaf explants to obtain plantlets of *U. tomentosa* and calli of both species. It is already known that the species can be produced by seed germination[32,33] and in the present study both species germinated completely within 60 days and were then maintained for six months in pots in a greenhouse, before transfer of the young plants to the field. After 24 months leaf samples were harvested and submitted to alkaloid analysis. The alkaloid profile of *U. tomentosa* plant matrix is shown in Figure 3(a) and that of *U. guianensis* in Figure 4(e). The total amount of alkaloid in the *U. tomentosa* leaf matrix (19.8 mg g^{-1}) was around four-fold higher than in the *U. guianensis* leaf matrix (4.43 mg g^{-1}) (Table 1). The higher content of alkaloid in *U. tomentosa* corresponds to the data already described for seed or stem stock cultivated Peruvian plants.[14,15] A low concentration of speciophylline (**1**) and the absence of uncarine F (**3**) was observed in both matrices, quite similar to the POA profile of the reference sample obtained from a wild Brazilian *U. tomentosa* specimen (Figure 2, 3(a) and 4(e), Table 1).

Although seeds can be used to propagate these *Uncaria* species, generally, it can be difficult because viability rapidly declines after dehiscing,[20,34] collection in the wild is not easy since flowering occurs normally only once a year and the species themselves are frequently located in areas of difficult access.[18,19] In addition a significant seasonal variation of the alkaloid profile and content in several parts of seed or stem stock cultivated specimens of both species was reported.[14,15] Thus, empirical examinations of *in vitro* cultivation methods for both *Uncaria* species were carried out in order to obtain rapid multiplication of those cultivars with morphologically homogeneous plants with desirable POA profiles and contents. Various techniques for the *in vitro* propagation of woody species are available and they can provide several identical individuals with economy of time and space.[35]

Callus inductions were obtained using previously developed optimized conditions that took into account the callus maximal growth rate.[36,37] In the search for these conditions the influence of four auxins [indolebutyric acid (IBA), 1-naphthylacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-amino-3,5,6-trichloropicolinic acid (Picloram)] at different concentrations were evaluated for both species in MS culture medium with 0.6% of agar and 3% of sucrose. The results showed that with Picloram supplement at 0.5 mg L^{-1} the best callus inductions and maximal growth rate for both species were obtained. NAA was unable to promote
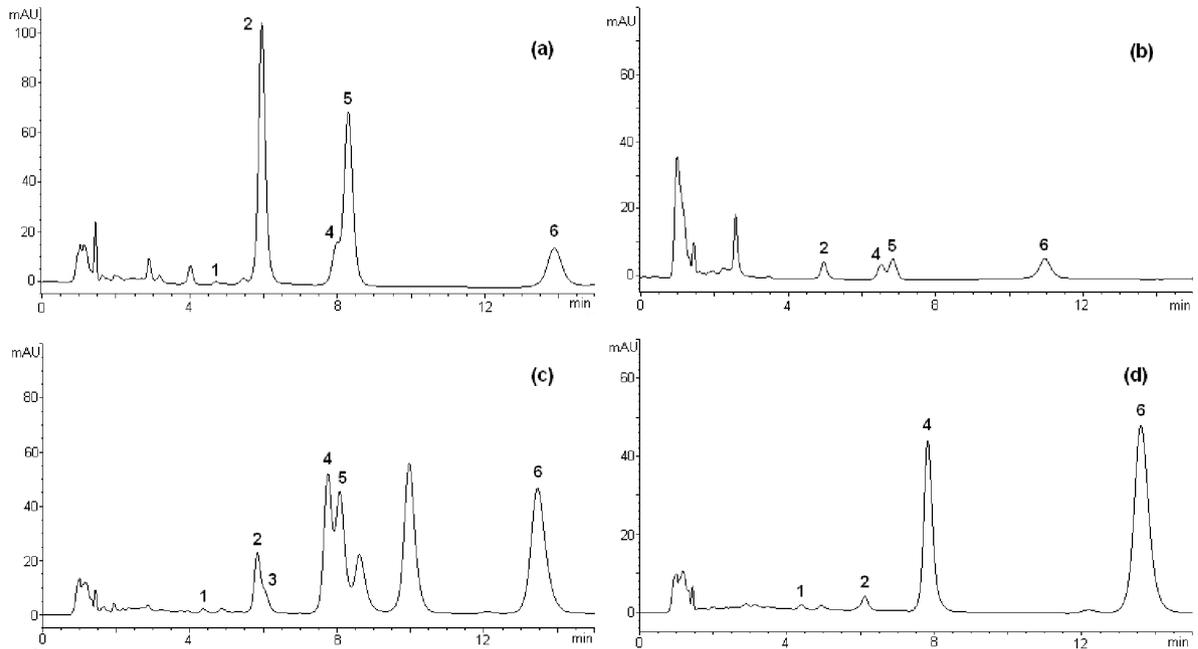
Figure 3. Pentacyclic oxindole alkaloid HPLC profiles of the *U. tomentosa* (UT) samples: **1**: Speciophylline; **2**: Mitraphylline; **3**: Uncarine F; **4**: Pteropodine; **5**: Isomitraphylline; **6**: Isopteropodine. **(a)** UT leaf matrix; **(b)** UT plantlets; **(c)** UT plantlets acclimatized for 30 days; **(d)** UT plantlets acclimatized for 45 days.
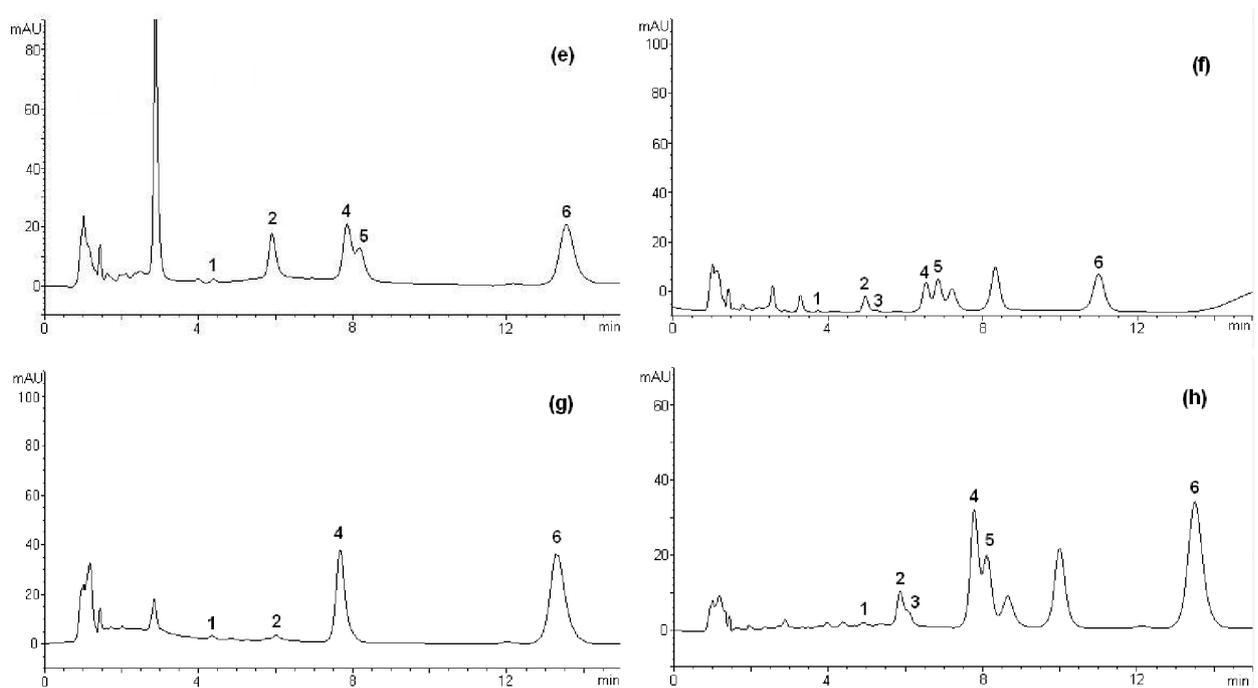
Figure 4. Pentacyclic oxindole alkaloid HPLC profiles of the *U. guianensis* (UG) samples: **1**: Speciophylline; **2**: Mitraphylline; **3**: Uncarine F; **4**: Pteropodine; **5**: Isomitraphylline; **6**: Isopteropodine. **(e)** UG leaf matrix; **(f)** UG plantlets; **(g)** UG plantlets acclimatized for 30 days; **(h)** UG plantlets acclimatized for 45 days.

induction in both species, 2,4-D promoted induction only in *U. guianensis* and IBA barely promoted a weak induction in *U. guianensis*. After the callus induction protocols for both species had been defined, fresh sets of the material were prepared and those cultivated for 30, 60 and 90 days were submitted to POA analyses. The results revealed no target alkaloids in the analyzed calli showing that the callus development conditions in spite of the good biomass

Table 1. Total alkaloid content and percentage of distribution of the pentacyclic oxindole alkaloids on the plant matrices, plantlets and acclimatized plantlets methanol extracts of *Uncaria tomentosa* and *Uncaria guianensis*

	Samples	Total alkaloids[a] /(mg/g ± 3%)	Percentage of distribution of POA[b]						Total percentage of POA[b]
			1	**2**	**3**	**4**	**5**	**6**	
U. tomentosa	(a)	19.8	0.46	26.6	-	4.02	17.3	12.6	61.0
	(b)	2.00	-	5.58	-	5.07	9.75	17.4	37.8
	(c)	16.3	0.48	6.11	0.50	14.1	13.7	23.3	58.2
	(d)	23.3	0.76	2.45	-	27.3	-	52.6	83.1
U. guianensis	(e)	4.43	0.41	6.92	-	11.2	6.57	20.4	45.5
	(f)	2.00	0.65	3.93	0.43	8.63	10.9	18.1	42.6
	(g)	8.30	0.27	1.49	-	21.3	-	34.3	57.4
	(h)	12.3	0.45	5.15	0.49	16.9	10.6	33.0	66.6

[a] *n*=3; [b] In area percent; POA: Pentacyclic Oxindole Alkaloids; (a) and (e): plant matrices; (b) and (f): plantlets; (c) and (g): plantlets acclimatized for 30 days; (d) and (h): plantlets acclimatized for 45 days; **1**: Speciophylline; **2**: Mitraphylline; **3**: Uncarine F; **4**: Pteropodine; **5**: Isomitraphylline; **6**: Isopteropodine.

production were still inappropriate for POA production. The influence of the auxins 2,4-D and indole-3-acetic acid (IAA) and sucrose concentration (supplemented with kinetin in MS culture medium) has been recently demonstrated in the production of the POA mitraphylline (**2**), pteropodine (**4**) and isopteropodine (**6**) by *U. tomentosa* cell suspension cultures.[17] The biosynthetic production of the POA seems to originate from the related indole alkaloids and both alkaloid types can occur together in *U. tomentosa* and *U. guianensis*.[14,15] The influence of auxins and the culture medium on the production of indole alkaloids in callus culture of *U. rhynchophylla* has been described[38] and clearly showed that callus induction and growth and alkaloid production were dissociated in some conditions. The conditions used in this study for obtaining calli of *U. guianensis* and *U. tomentosa*, although they produced non-satisfactory callus culture for POA production, did emphasize the different behaviors of the two plant species studied. Some other differences related to alkaloid contents and pharmacological activities have been mentioned by authors such as Laus and Kepligler[15] and Sandoval *et al.*,[39] leading to an interesting discussion about their similar use in folk medicine.

As an alternative, the species were micropropagated by developing plantlets of both species using conditions previously optimized for a high percentage of germination and maximal growth. The search for these optimized conditions was made by empirical variation of different factors such as culture medium, sucrose concentration, explant inoculation position, etc.[37,40] In this previous work it was pointed out that while embryos of *U. guianensis* were excellent sources for micropropagation purposes, for *U. tomentosa* their use under several different conditions did not lead to *in vitro* germination. On the other hand, leaf explants of *U. tomentosa* have been successfully used. Thus, after the *in vitro* propagation

protocols for both species had been defined, fresh sets of plantlets were produced in order to analyze their POA profiles. The plantlets obtained from both species in the optimized conditions showed the same alkaloid contents (2.00 mg g⁻¹, Table 1) but some differences in the POA profiles. They showed a low concentration of speciophylline (**1**) and absence or a low concentration of uncarine F (**3**) like the plant matrix POA profiles and predominance of isopteropodine (**6**) (Figure 3(b) and 4(f), Table 1). The total percentage (area percent) of POA in *U. tomentosa* plantlets was 37.8% while in *U. guianensis* plantlets it was 42.6% (Table 1). In addition the *U. guianensis* plantlet alkaloid profile showed non-identified alkaloids with a retention time between isomitraphylline (**5**) and isopteropodine (**6**) (Figure 4(f)). Subsequently the plantlets were acclimatized in a greenhouse in the same conditions of the plant matrices in order to accompany their behavior[37, 41] as well as to analyze possible changes in their POA profiles. Leaves from both species at two points of the acclimatization period showed a remarkable improvement in the total alkaloid content, especially for *U. tomentosa* (Table 1), as well as interesting alkaloid profiles. The total alkaloid amounts found in the leaves of plantlets of *U. tomentosa* acclimatized for 30 and 45 days were 16.3 and 23.3 mg g⁻¹ respectively which is quite similar to the amount found for the *U. tomentosa* leaf matrix (19.8 mg g⁻¹). However for *U. guianensis* the amounts found were significantly higher than the amount found for its matrix (4.43 mg g⁻¹): 8.30 mg g⁻¹ for 30 days of acclimatization and 12.3 mg g⁻¹ for 45 days of acclimatization (Table 1). The chromatograms revealed for *U. tomentosa* leaves collected on the 30th day of acclimatization the presence of the six POA markers together with the non-identified alkaloids with retention times between isomitraphylline (**5**) and isopteropodine (**6**) (Figure 3(c)) and for the leaves collected 15 days later five POA with predominance of

pteropodine (**4**) and isopteropodine (**6**) (Figure 3(d), Table 1). However for *U. guianensis* the leaves collected at the 30th day of acclimatization showed four POA with the predominance of pteropodine (**4**) and isopteropodine (**6**) (Figure 4(g), Table 1) and those collected 15 days later all six oxindole alkaloid markers together with the non-identified alkaloids with retention times between isomitraphylline (**5**) and isopteropodine (**6**) (Figure 4(h)). Laus *et al.*[14] have reported for the seasonal screening of indole and oxindole alkaloids in several parts of seed or root stock of cultivated Peruvian species of *U. tomentosa* collected in different locations, a higher content of alkaloids in young leaves than in mature leaves and a significant seasonal variation of the alkaloid content. For cultivated and wild Peruvian species of *U. guianensis*[15] the comparison of the alkaloid content between young and mature leaves did not show a significant variability, but was limited to mitraphylline (**2**) and isomitraphylline (**5**) as the only POA in all samples. All these previous results, added to those found for our plant materials, confirm the complexity and variability of POA profiles for *in vivo* and *in vitro* cultivated specimens of both studied species. Since seed germination presents the inconveniences already mentioned and a germination time that may vary depending on the region in which the plant is growing, and that stem stock germination, due to the high humidity[20,34] it requires, also presents some limitations, our results show the feasibility of the *in vitro* propagation methodology for a quicker and controlled cultivation of *U. tomentosa* and *U. guianensis*, producing suitable young plants to be transferred to the field in a shorter period of time with no limitation of season or scale. This is, to the best of our knowledge, the first report of plantlet propagation protocols for the South-American species of *Uncaria* with the additional characterization and quantification of their POA. They also provide data on alkaloid composition of Brazilian *Uncaria* species which are currently scanty. The unidentified compounds produced in same micropropagated samples will be further accomplished. These results proved that micropropagation is a feasible tool for the growth of the species, and may thus be important for their commercialization and for their conservation as a forest resource.

Acknowledgments

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