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In vitro shoot and root organogenesis, plant regeneration and production of tropane alkaloids in some species of *Schizanthus*

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Abstract

A rapid in vitro propagation system leading to formation of shoots from callus, roots, and plantlets was developed for *Schizanthus hookeri* Gill. (Solanaceae), an endemic Chilean plant. The genus *Schizanthus* is of particular interest due to the presence of several tropane alkaloids. So far, in vitro propagation of species of this genus has not been reported. Propagation of *S. hookeri* consisted of two phases, the first one for callus initiation and shoot formation and the second for rhizogenesis and plantlet regeneration. From a single callus that rapidly increased in cell biomass (from ~50 mg to ~460 mg/culture tube [25 × 130 mm] in 60 days) in the presence of 2.69 μM NAA and 2.22 μM BA, more than 10 shoots/callus explant were formed. From the latter, approx. twenty plantlets formed after 90–110 days shoot subculture in medium devoid of growth regulators that favored root formation. Ten alkaloids ranging from simple pyrrolidine derivatives to tropane esters derived from angelic, tiglic, senecioic or methylmesaconic acids were obtained from in vitro regenerated plantlets. One of them, 3α-methylmesaconyloxytropane was not previously described. The same growth conditions, as well as other growth regulator levels tested, were required to induce callus and root formation in *S. grahamii* Gill. Root organogenesis occurred despite a high level of BA vs. NAA used, (i.e., 4.44 μM BA and 0.54 μM NAA); however no shoot formation was achieved. In the case of *S. tricolor* Grau et Gronbach, only callus formation was obtained in the presence of various growth regulators.

Keywords: *Schizanthus hookeri*; *S. grahamii*; *S. tricolor*; Solanaceae; Organogenesis; In vitro propagation; Tropane alkaloid content

1. Introduction

The genus *Schizanthus* (Solanaceae), which belongs to the tribe Salpiglossideae, includes 12 species (Grau and Gronbach, 1984; Marticorena, 1990; Grau, 1992) endemic to the south western slopes of the Chilean Andes. Previous chemical studies on *Schizanthus* have shown that this genus accumulates a number of tropane-derived alkaloids such as hydroxytropane esters, dimeric tropane diesters, cyclobutane tricarboxylic acid triesters and pyrrolidine alkaloids (San-Martín et al., 1980, 1987; Gambaro et al., 1982, 1983; De la Fuente et al., 1988; Muñoz et al., 1991, 1994; Muñoz, 1992; Muñoz and Cortés, 1998; Griffin and Lin, 2000). The well known effects of tropane alkaloids as anticholinergic, antiemetic, parasympatholytic and anesthesic agents (Hashimoto and Yamada, 1992; Fodor and Dharanipragada, 1994) have stimulated considerable interest during the last decades on the biosynthetic pathway leading to tropane alkaloids (Lounasmaa and Tamminen, 1993).

Schizanthus hookeri, *S. grahamii* and *S. tricolor* (formerly *S. litoralis*) are three Andean species that accumulate several tropane bases, in particular schizanthines (San-Martín et al., 1987; De la Fuente et al., 1988; Muñoz

Abbreviations: BA, benzyladenine; IBA, indole-3-butyric acid; NAA, naphthaleneacetic acid; MS, Murashige and Skoog medium.

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et al., 1991; Muñoz and Cortés, 1998), and grahamine isolated from *S. grahamii* (Hartmann et al., 1990).

Many efforts have been made to develop economically feasible methods for the production of tropane alkaloids by applying cell culture techniques (Zárate et al., 1997; Christen, 2000; Khanam et al., 2001). The main efforts toward the industrial preparation of tropane alkaloids by cell culture methods have concentrated on hyoscyamine and scopolamine (Lounasmaa and Tamminen, 1993; Oksman-Caldentey and Hiltunen, 1996; Oksman-Caldentey and Arroo, 2000).

The biosynthesis of tropane alkaloids has been extensively studied over the last decades (Robins et al., 1994). In particular, it has been demonstrated that the site of biosynthesis is the root, the alkaloids being translocated from the roots to the aerial parts of the plants (Hashimoto et al., 1991). Despite considerable efforts to produce secondary metabolites by undifferentiated plant cell cultures, it has become increasingly apparent that having a degree of morphological organization present greatly enhances the likelihood of successful product formation in vitro (Robins and Walton, 1993).

The study of alkaloids in *Schizanthus* is fairly recent and in vitro morphogenic aspects and regeneration of these species from cell cultures have not been reported so far. Furthermore, there is no information on the secondary metabolite production of in vitro cultures in these species and its variation according to explant-types and growth regulators. The aim of this work was to evaluate the in vitro morphogenic potential of three *Schizanthus* species and the regeneration of selected plants. A second aim of the work was to evaluate the alkaloid patterns produced by the various tissues, organs and plantlets of *Schizanthus* developed under in vitro conditions.

2. Results and discussion

2.1. In vitro propagation

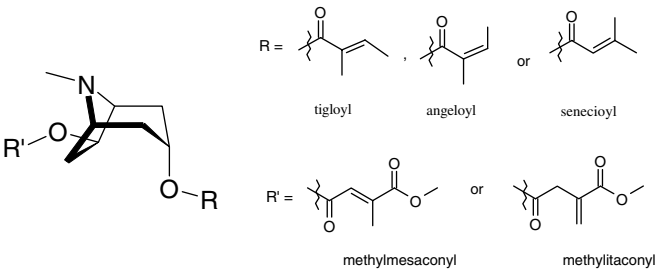
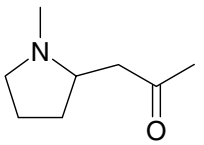
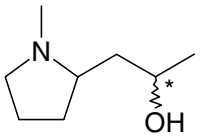
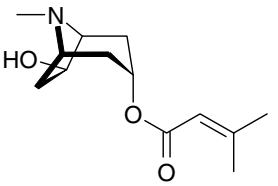
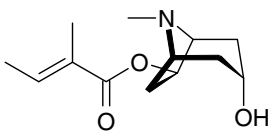
Callus initiation was the first and generalized response observed in internodal explants of all the species examined. Intense cell proliferation on the surface at the cut edges of the sections, leading to calli, was observed with high frequency after a period of 7–10 days in culture. The mass of the calli increased rapidly and they gradually turned green with the exception of *S. tricolor* whose calli turned brown after a period of 2–3 weeks. Shoot organogenesis was first observed after 2 months, occurring profusely in the calli of *S. hookeri* in the initiation medium supplemented with 2.69 μM NAA and 2.22 μM BA (Table 1) leading to several plantlets in a single culture tube. After the first subculture, multiple new shoots formed, developing roots and plantlets after 4–5 weeks in the same MS medium but devoid of sucrose and growth regulators. However, the same conditions, including treatment with 2.69 μM NAA and 2.22 μM BA did not trigger shoot formation in *S. grahamii* leading only to intensive callus growth. Although the calli developed remarkably dense green spots on the surface, these outgrowths never developed into shoots. However, in subculture the calli formed roots (more than 5/explant) in the presence of 0.54 μM NAA and 4.44 μM BA. Less intensive callus growth was observed with *S. tricolor*. In comparison with the other

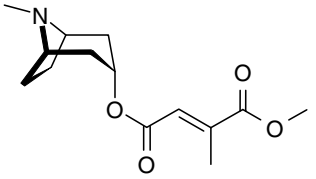
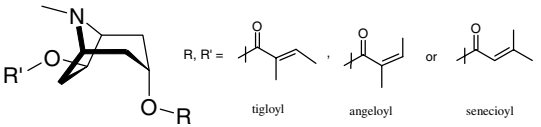
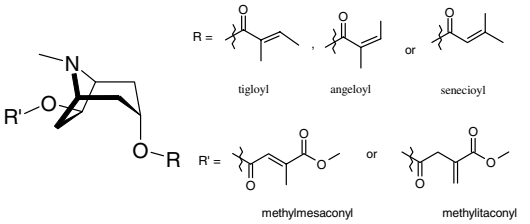
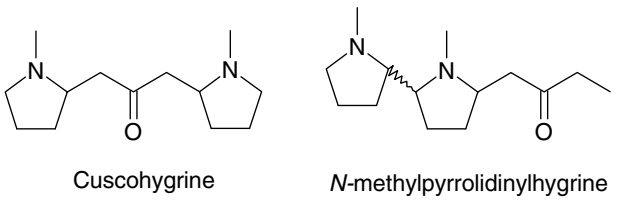
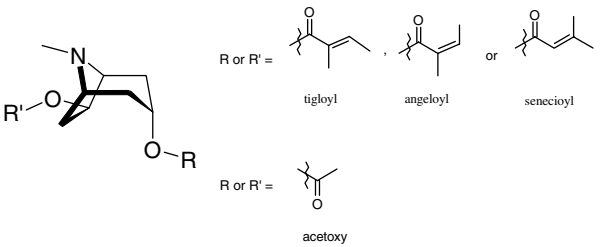
Table 1
Morphogenic responses observed in 3 *Schizanthus* species after 2 months in culture and subculture[a]

Species	Growth regulators NAA BA (μM)	Callus initiation (%) after 30 days[b]	Callus FW (mg) after 60 days (aver.)	Morphogenic responses after 60 days	Morphogenic responses after 1st subculture (results after 30–50 days)
S. hookeri	2.69 2.22	100	460.2	Green callus forming new shoots (approx. 10 shoots/callus). Plantlet regeneration	Shoot elongation, root formation and regeneration of plantlets in medium devoid of growth regulators and sucrose
	5.37 2.22	100	365.3	Green callus with roots only	Intense callus growth in presence of 26.85 μM NAA and 0.44 μM BA
S. grahamii	2.69 2.22	100	1257.0	Dense green spots on basal callus tissue, no shoots or roots	Root formation and callus growth in presence of 0.54 μM NAA and 4.44 μM BA. Green callus; no shoots
	5.37 2.22	100	594.0	Green spots on basal callus, no shoots or roots	Root formation and callus growth in presence of 0.54 μM NAA and 4.44 μM BA. No shoots
S. tricolor	2.69 2.22	75	175.0	No morphogenic responses; little callus growth; brown callus	Brown callus in presence of 26.85 μM NAA and 0.44 μM BA
	5.37 2.22	80	160.3	No morphogenic responses, little callus growth; brown callus	Brown callus in presence of 0.54 μM NAA and 4.44 μM BA.

[a] All treatments started with 20 tubes per culture using internodal sections.
[b] Internodal sections initiating callus.

Table 2
Characteristics of tropane alkaloids identified in in vitro plantlets and calli of *Schizanthus hookeri*, *S. tricolor* and *S. grahamii*

Schizanthus species	Alkaloid	Rt (min)	[M^+] *m/z*	Peak area % (average values)	Reference mass spectrum	Structure
S. hookeri						
Callus	Tigloyloxy- or angeloyloxy- or seneciolyoxy- and methylmesaconyloxy or methylitaconyloxytropane	36.09	365	–	–	
Regenerated plantlets	Hygrine	7.43	141	4.16	+	
	Hygroline A (or B)	7.66	143	2.46	+	
	Hygroline B (or A)	8.00	143	0.97	+	
	3α-Senecioyloxy-7β-hydroxytropane	25.57	239	0.95	+	
	3α-Hydroxy-7β-tigloyloxytropane	26.13	239	0.40	+	

	New alkaloid 3α-methymesaconyloxytropane	27.1	267	1.41	+	
	3,7-Disubstituted tropane alkaloid with tiglic or angelic or senecioic acids	35.7	321	2.93	–	
	Tigloyloxy- or angeloyloxy- or seneciolyoxy- and methylmesaconyloxy- or methylitaconyloxytropane	36.12	365	1.16	–	
	Tigloyloxy- or angeloyloxy- or seneciolyoxy- and methylmesaconyloxy- or methylitaconyloxytropane	37.00	365	0.97	–	
	Tigloyloxy- or angeloyloxy- or seneciolyoxy- and methylmesaconyloxy- or methylitaconyloxytropane	37.23	365	1.86	–	
S. tricolor Callus	Cuscohygrine or*N*-methylpyrrolidinylhygrine	25.9	224	–	+	
S. grahamii Callus	Acetoxy and tigloyloxy or angeloyloxy or senecioyloxytropane	30.82	281	–	–	

species the calli of *S. tricolor*, showed less chloroplast formation turning brown within a month. Also, contrary to the other species, the calli of *S. tricolor* were unable to recover in subculture under various levels of growth regulator combinations tested (data not shown). Growth and morphogenic responses of these three species are summarized in Table 1.

2.2. Alkaloid determination

Preliminary determination of tropane alkaloids in tissues developed in vitro by weighing indicated their presence in callus tissue as well as in the regenerated plantlets. While the level detected in calli of the three species under study was similar (i.e., 0.9, 1.1, and 1.6 mg g^{-1} dry weight), this content increased fivefold in regenerated plantlets of *S. hookeri* (5.6 mg g^{-1} dry weight).

2.3. Alkaloid identification

Different alkaloids were detected in the various plant parts studied. Only one alkaloid was detected in the calli of *S. hookeri* (Table 2). The $[M]^{+\cdot}$ at 365 thomsons (Th), together with prominent ions at 238, 222, 138, 122 and 94 Th (base peak) suggested a 3,6- or 3,7-disubstituted tropane nucleus of molecular formula $C_{19}H_{27}NO_6$ esterified with $C_5H_8O_2$ (tiglic, senecioic or angelic acid) and $C_6H_8O_4$ (methylmesaconic or methylitaconic acid) moieties. However, in the absence of reference compound, its unambiguous identification was not possible and only tentative assignments were made.

From the in vitro regenerated plantlets of *S. hookeri*, ten alkaloids were detected by GC–MS (Table 2) including hygrine and hygrolines A and B. The latter compounds are not discussed in more details as they are not tropane alkaloids and are frequently encountered in solanaceous plants. According to the fragmentation patterns, seven alkaloids proved to belong to the tropane series. Two isomers of 239 Daltons (Da) were identified by comparison of their retention indices (Bieri et al., 2006). The first one was identified as 3α-senecioyloxy-7β-hydroxytropane ($I = 1866.2$) and the second one as 3α-hydroxy-7β-tigloyloxytropane ($I = 1894.0$). Another alkaloid of 267 Da was detected ($I = 1944.8$). This compound has not been previously identified and is a new alkaloid. Its characterization was a difficult task due to the very small amount present in the extract. According to its fragmentation determined by electron impact MS, two possible structures were suggested: 3α-methylmesaconyloxytropane (**1**) or 3α-methylitaconyloxytropane (**2**). These two compounds are configurational isomers (Fig. 1). In order to assign unambiguously a structure to this new alkaloid, it was necessary to synthesize both possible isomers.

Fig. 2(a) and (b) show the mass spectra of the synthetic 3α-methylmesaconyloxytropane ($I = 1942.4$) and 3α-methylitaconyloxytropane ($I = 1893.0$), respectively. Fig. 2(c) shows the mass spectrum of the compound at 267 Th

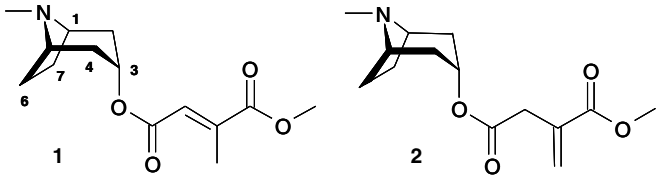


Fig. 1. Structures of the two isomers of 267 Da, 3α-methylmesacoyloxytropane (**1**) and 3α-methylitaconyloxytropane (**2**).

detected in the regenerated plant extract which matched isomer (**1**). This compound was also detected in the stem–bark extract of the field-grown plants.

In the same extract, other alkaloids were detected but could not be identified unambiguously and among them, an isomer of 321 Da (Table 2). The fragmentation pattern showed that it was a 3,6- or 3,7-disubstituted tropane alkaloid with tigloyl, senecioyl or angeloyl moiety. Three other isomers of 365 Da were also detected. Fragmentation patterns indicated that they were 3,6 - or 3,7-tropane diesters with tigloyl, senecioyl or angeloyl and methylmesaconyl or methylitaconyl moieties. However, for all of them, lack of reference material precluded their unambiguous identification. In order to compare the alkaloid pattern of the regenerated plantlets with that of the normal plants, GC–MS analysis of the stem–bark extract of field grown *S. hookeri* led to the identification of the following tropane alkaloids: hygrine; hygrolines A and B; tropine, tropinone, 3,7-tropane-diol, 3α-senecioyloxytropane, 3α-senecioyloxy-7β-hydroxytropane, 3α-hydroxy-7β-angeloyloxytropane, 3α-hydroxy-7β-tigloyloxytropane, 3α-hydroxy-7β-senecioyloxytropane, 3α-methylmesaconyloxytropane and 6 isomers at 365 Da. In the absence of reference compounds, the identification of these isomers was not possible. The presence of the 3α-senecioyloxytropane derivative in the stem–bark of *S. hookeri* was confirmed thanks to the linear retention index calculated for the analyte detected in the extract ($I = 1661.2$) and from a reference compound ($I = 1661.4$). Previously, (San-Martín et al., 1980; Gambaro et al., 1982, 1983) the following alkaloids in *S. hookeri* were identified: The diastereoisomeric hygrolines, 3α-hydroxytropane (tropine), 3α-senecioyloxytropane, 3α,-7β-dihydroxytropane, 3α-hydroxy-7β-tigloyloxytropane, 3α-hydroxy-7β-angeloyloxytropane and 3α-senecioyloxy-7β-hydroxytropane.

In the calli of *S. tricolor*, one alkaloid only, cuscohygrine or *N*-methylpyrrolidinylhygrine (224 Da) was detected. Similarly, in the green calli of *S. grahamii* (Table 2), one alkaloid only was identified (281 Da). Its fragmentation pattern showed that it is a 3,6- or 3,7-disubstituted tropane alkaloid with acetic and tiglic, senecioic or angelic substituents as esterifying acids. Previous chemical work on *S. grahamii* and *S. tricolor* aerial parts have shown that both species accumulate tropane alkaloids, i.e hydroxytropane esters, dimeric tropane diesters of methylmesaconic and methylitaconic acids, cyclobutane tricarboxylic triesters and ferulamides (San-Martín et al., 1987; De la Fuente

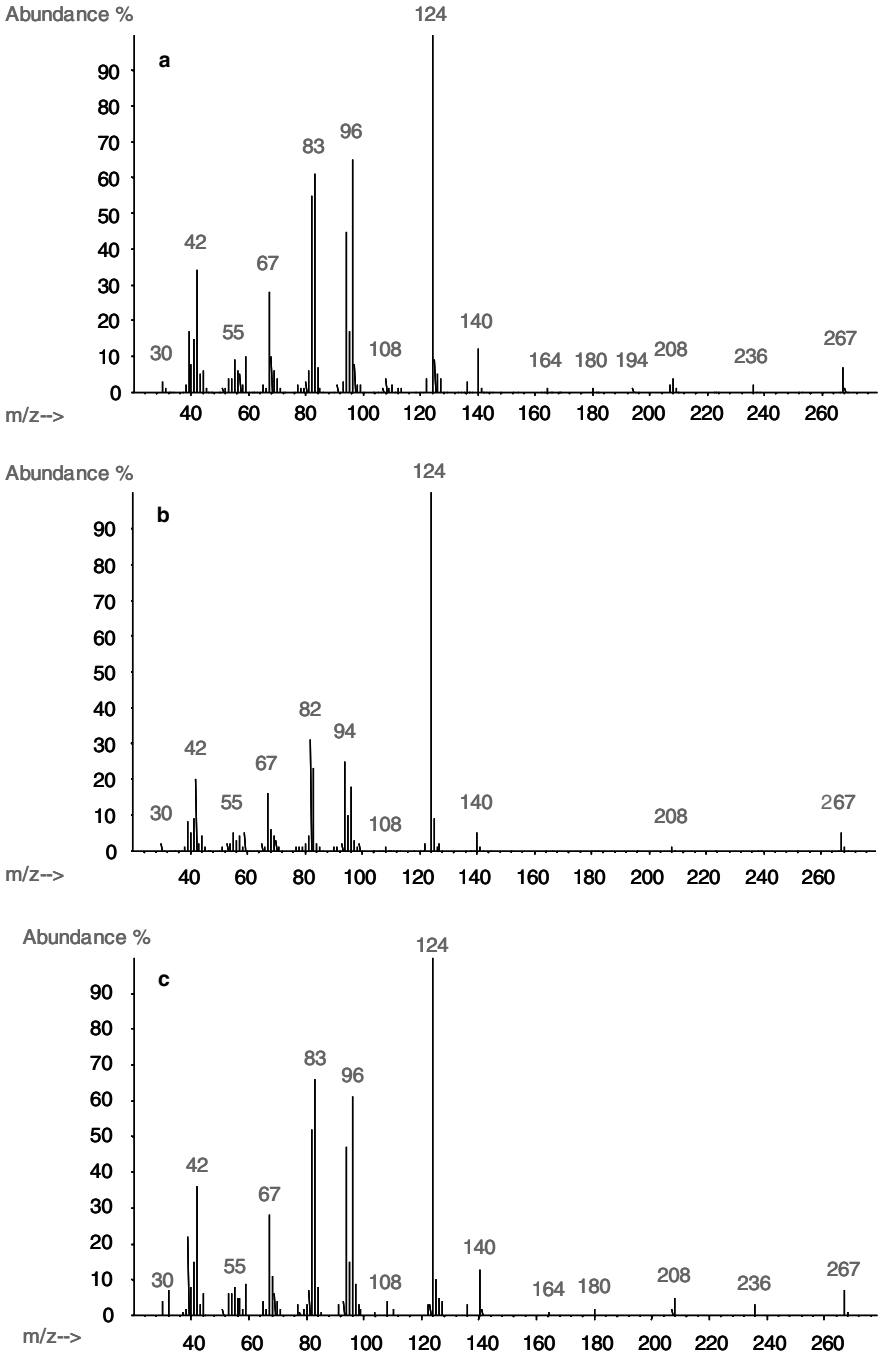


Fig. 2. MS spectra of: (a) 3α-methylmesaconyloxytropane (**1**); (b) 3α-methylitaconyloxytropane (**2**); (c) alkaloid of 267 Th present in the regenerated plantlet extract of *S. hookeri*.

et al., 1988; Hartmann et al., 1990; Bieri et al., 2006; Muñoz et al., 1996).

2.4. Synthesis of 3α-methylmesaconyloxytropane (*1*)

This alkaloid of 267 g/mol (**1**) was obtained via a three-step synthesis (Fig. 3). First, 2-methylmesaconic acid (**4**) was obtained by Wittig reaction as the condensation product between the commercially available methyl 2-bromopropionate (**3**) and glyoxylic acid monohydrate (Zumbrunn et al., 1985; Wolff et al., 2002). The synthetic strategy was to perform the selective formation of the methyl group on C-2 and carboxylic functional group on C-4 with *E* double bond configuration. The identity of the product was confirmed by HMBC NMR experiment.

Methylmesaconic acid (**4**) was then converted to methylmesaconic acid chloride (**5**) by treatment with thionyl chloride. Finally, the 3α-methylmesaconyloxytropane (**1**) was obtained by the addition of 3α-tropine to methylmesaconic acid chloride (**5**).

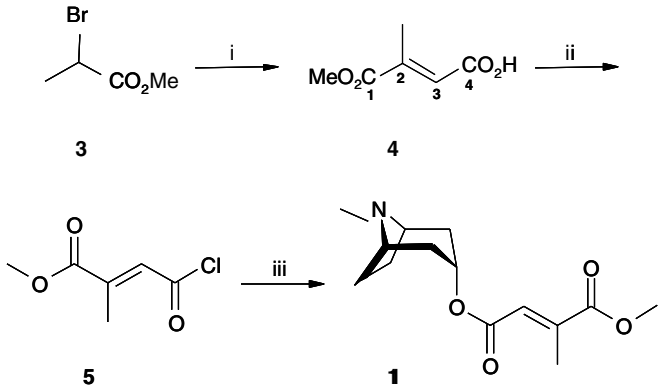


Fig. 3. Synthesis of 3α-methylmesaconyloxytropane (**1**). Reagent and conditions: (i) (1) PPh_3, dry CH_3CN, N_2, 65 °C, overnight, (2) glyoxylic acid monohydrate, *N*-diisopropylethylamine, dry CH_3CN, N_2, 0 °C 2 h, room temp. overnight; (ii) Thionyl chloride, Ar, reflux; (iii) 3α-Tropine, Ar, 105 °C, 4 h, room temp. overnight.

3. Experimental

3.1. Plant material

Schizanthus plants growing in the wild were collected by O. Muñoz from November 2002 to January 2003 in the following sites: *S. hookeri*, Lagunillas at 2100 m altitude (SQF 22231), *S. grahamii*, Rengo, Laguna Los Cristales at 2200 m (SQF 0015), *S. tricolor*, Papudo at 10 m (SQF 0017); voucher specimens are kept in the Herbario de la Escuela de Química y Farmacia, Santiago. All samples were identified by Dr. F. Pérez, with the only exception of *S. grahamii* (J. San-Martín, Universidad de Talca). Internodes from plants collected in the field were washed and their surface disinfected with the fungicides Captan® (Micro Flo Company LLC, Memphis, USA) and Benlate® (Du Pont and Co. Inc. Nemours, France) (0.2% each) for 30 min under constant shaking, washed with sterile distilled H_2O and sterilized with 3% NaOCl for 5 min. Internodal sections of approx. 1 cm (~50 mg), were placed in tubes (25 × 130 mm) containing 12.5 ml MS liquid nutrient medium (Murashige and Skoog, 1962) supplemented with 3% sucrose on Whatman Nr 1 paper bridges in the presence of NAA at 2.69 and 5.37 μM combined with 2.22 μM BA, according to the best responses obtained in preliminary work (data not shown). Various growth regulator levels were used in subculture (0.54 μM NAA combined with 4.44 μM BA, or 0.49 μM IBA); supplemented with agar, but with poorer results. All plant tissue culture media, salts, vitamins, growth regulators and agar were obtained from Sigma (St. Louis, USA). Explants were maintained in a light regime of 14 h at 48 μmol m^{-2} s^{-1} provided by daylight fluorescent lamps (Philips TLT 40W/54 R.S.) and a temperature of 22 ± 1 °C.

Calli of *S. grahamii* and *S. tricolor* reaching approx. 2.0 cm in diameter after 2 months were transferred to the same MS medium in the presence of various combinations of NAA and BA to induce shoot and/or root formation (1st subculture). In the case of *S. hookeri* calli, which produced shoots, roots and plantlets directly in the first culture phase, further subculturing was carried out in MS medium devoid of growth regulators and sucrose. Later on, plantlets were transferred to non-sterile conditions using a substrate composed of equal volumes of sand/vermiculite/organic soil in growth chambers for 1-month acclimatization followed by transfer to the greenhouse.

3.2. Chemicals

Ethyl acetate, diethyl ether, methanol, dichloromethane, chloroform, hexane, $NaHCO_3$, Na_2SO_4, $MgSO_4$, HCL, ammonia and thionyl chloride were purchased from Fluka (Buchs, Switzerland). Acetonitrile was from SDS (Peypin, France).

3.3. Extraction of tropane alkaloids

Tropane alkaloid extraction was performed as previously described (Kamada et al., 1986; Zárate et al., 1997). Material derived from in vitro culture was lyophilized for 24 h, homogenized and extracted with a mixture of $CHCl_3$:MeOH:NH_3 15:15:1. Ten ml per 100 mg of sample was sonicated for 10 min and left at room temperature for 1 h. After filtration, the plant material was washed with $CHCl_3$ (2 × 1 ml) and the pooled filtrate was evaporated to dryness. Thereafter, $CHCl_3$ (5 ml) and 1 N H_2SO_4 (2 ml) were added to the residue and mixed thoroughly for alkaloid extraction. The organic phase was discarded and the aqueous phase was made basic (pH 10) with conc. ammonia. Alkaloids were extracted with $CHCl_3$ (1 × 2; 2 × 1 ml). The combined organic phases were filtered after addition of anhydrous Na_2SO_4, filtered and evaporated to dryness (alkaloid crude extract). The extracts were taken up in MeOH (1 ml) (HPLC grade, Merck, Darmstadt, Germany), filtered through a 0.45 μm filter membrane (Lida Manufacturing Corp.) and kept at −20 °C until required for analysis.

3.4. Gas chromatography–mass spectrometry

GC–MS analyses were carried out using a Hewlett–Packard chromatograph 5890 series II coupled to a HP 5972 mass-selective detector (Agilent Technologies, Palo Alto, USA). The MS detector was used in the electron impact ionization (EI) mode with an ionization voltage of 70 eV. Mass spectra were recorded in the range 30–600 Th at 1.3 scan/s and the MS transfer line was set at 280 °C. The capillary column (HP5-MS 30 m × 0.25 mm i.d., 0.25 μm film thickness) was used with He as carrier gas under the following conditions: an initial oven temperature of 70 °C was maintained for 1 min then linearly increased at 5 °C/min to a final temperature of 285 °C, and held at this temperature for 15 min. A sample volume of 1 μl was injected in the splitless mode into a laminar liner at 250 °C using a fast HP 6890 series autosampler.

Retention indices were determined in the split mode (15:1) and calculated using a linear gradient temperature: 70 °C (0 min) linearly increased at 5 °C/min to a final temperature of 285 °C and held at this temperature for 15 min.

3.5. NMR

^{1}H and ^{13}C NMR, COSY, NOESY, HMBC and HSQC spectra were recorded in $CDCl_3$ using a 500 MHz Bruker DRX instrument (Bruker, Dübendorf, Switzerland) equipped with a QNP probehead. Chemical shift values (δ) were reported in parts per million (ppm) related to tetramethylsilane as internal standard and coupling constants (J) are given in Herz.

3.6. HRMS

High resolution mass spectra were obtained on a QStar XL TOF mass spectrometer (MDS Sciex, Concord, Ontario, Canada) by direct injection (5 ng/ml CH_3CN + 0.1% HCO_2H) using NanoMate 100 (Advion BioSciences, Ithaca, NY, USA).

3.7. IR

IR spectra were recorded on a Perkin–Elmer FT-IR spectrometer (Boston, USA).

3.8. Synthesis and spectral data

2-Methylmesaconic acid (**4**): To a solution of methyl-2-bromopropionate (**3**) (4.183 g, 24.3 mmol) in dry CH_3CN (60 ml) was added triphenylphosphine (5.66 g, 21.6 mmol, 0.9 eq.), in a 100 ml flask. After stirring at 65 °C overnight, *N*-diisopropylethylamine (3.82 ml, 21.9 mmol, 0.9 eq.) and glyoxylic acid monohydrate (2.077 g, 21.9 mmol, 0.9 eq.) dissolved in dry CH_3CN (10 ml) were added to the reaction mixture at 0 °C. The solution was further stirred at 0 °C for 2 h and at room temperature overnight. Half of the solvent was removed under reduced pressure and EtOAc (20 ml) was added. The resulting solution was washed with saturated aqueous $NaHCO_3$ (3×40 ml). The combined aqueous layers were extracted with EtOAc (2×20 ml), made acid (pH 1–2) at 0 °C with conc. HCl and extracted with EtOAc (3×30 ml). The combined organic layers were dried over $MgSO_4$, filtered and evaporated to dryness, yielding a pale yellow solid (1.487 g, 43%, $R_f = 0.30$, ethyl acetate:hexane 7:3), which was used in the next reaction without further purification. ^{1}H NMR ($CDCl_3$): δ 2.30 (3H, *d*, $J = 1.45$ Hz, H-5), 3.82 (3H, *s*, H-6), 6.79 (1H, *q*, $J = 1.5$ Hz, H-3), 11.36 (1H, *s*, OH); ^{13}C NMR ($CDCl_3$): δ 14.6 (C-5), 52.8 (C-6), 126.1 (C-3), 145.9 (C-2), 167.4 (C-1), 171.3 (C-4).

3-Methylmesaconic acid chloride (**5**): 1.43 g (9.92 mmol) of 2-methylmesaconic acid (**4**) was added to a two-necked round-bottomed flask equipped with a magnetic stirrer and a condenser. Thionyl chloride (3.6 ml, 49.6 mmol, 5 eq.) was then added under argon. The reaction mixture was heated to reflux (70 °C) during 2 h under stirring and argon. The excess of thionyl chloride was removed by distillation. The crude product was purified by distillation at reduced pressure yielding a colorless oil weighting 1.069 g (66%). ^{1}H NMR ($CDCl_3$): δ 2.23 (3H, *d*, $J = 1.64$ Hz, H-4), 3.81 (3H, *s*, H-6), 6.98 (1H, *q*, $J = 1.55$ Hz, H-2); ^{13}C NMR ($CDCl_3$): δ 14.7 (C-4), 52.8 (C-6), 131.2 (C2), 146.5 (C-3), 164.7 (C-5), 166.1 (C-1).

3α-Methylmesaconyloxytropane (**1**): 0.773 g (5.47 mmol, 1 eq.) of 3α-tropine was added to 0.890 g of (**5**). The mixture was heated to 105 °C while stirring under Ar during 4 h and then left at room temperature overnight. The reaction mixture was washed with Et_2O (3×25 ml). The aqueous layer was made basic with $NaHCO_3$ (pH 8–9) and extracted with CH_2Cl_2 (3×25 ml). The organic layers were dried over Na_2SO_4, filtered and the solvent removed. The crude brown oil was purified by silica CC (MeOH:$CHCl_3$:NH_3 (25%) 10:90:1.5) $R_f = 0.4$, yielding 0.396 g (27%); IR: ν_{max} (KBr) cm^{-1}: 3426, 2951, 1716, 1646, 1436, 1359, 1341, 1266, 1203, 1115, 1063, 1036, 983, 808, 774; GC–EI–MS (R_t 26.90 min, $I_{PT} = 1942.4$) 70 eV: m/z 267 $[M]^+$ (7), 236 (3), 208 (4), 140 (14), 124 (100%), 108 (4), 96 (67), 94 (53), 83 (63), 82 (56), 67 (28), 59 (18), 42 (35); ^{1}H NMR ($CDCl_3$): δ 1.72 (2H, *d*, $J = 14.7$ Hz, H_{eq}-2, H_{eq}-4), 1.92 (2H, *m*, H_{exo}-6, H_{exo}-7), 2.00 (2H, *m*, H_{endo}-6, H_{endo}-7), 2.16 (2H, *dt*, $J = 15.02$ Hz, $J = 4.25$ Hz, H_{ax}-2, H_{ax}-4), 2.24 (3H, *s*, H-8), 2.26 (3H, *d*, $J = 1.5$ Hz, H-12), 3.08 (2H, *s*, H-1, H-5), 3.78 (3H, *s*, H-14), 5.04 (1H, *t*, $J = 5.35$ Hz, H-3), 6.71 (1H, *q*, $J = 1.5$ Hz, H-10); ^{13}C NMR ($CDCl_3$): δ 14.2 (C-12), 25.6 (C-6, C-7), 36.5 (C-2, C-4), 40.4 (C-8), 52.6 (C-14), 59.6 (C-1, C-5), 67.7 (C-3), 127.2 (C-10), 143.3 (C-11), 165.2 (C-9), 167.6 (C-13); HRMS-CI m/z calculated for $C_{14}H_{22}NO_4$ 268.1543 $[M + H]^+$, found 268.1554.

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