Secondary Metabolite Content in *Fabiana imbricata* Plants and *in vitro* Cultures

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A rapid *in vitro* propagation system leading to the formation of shoots, calli, roots, cell suspensions and plantlets was developed for the Andean medicinal plant *Fabiana imbricata* (Solanaceae). Massive propagation of shoots and roots was achieved by the temporary immersion system (TIS), morphogenesis and maintenance of cell suspensions by standard *in vitro* culture techniques. Oleanolic acid (OA), rutin, chlorogenic acid (CA) and scopoletin content in aerial parts of wild growing *Fabiana imbricata* plants as well as in plantlets regenerated *in vitro*, callus cultures, cell suspensions and biomass, obtained by the TIS system was assessed by HPLC.

On a dry weight basis, the OA content in the aerial parts of the plant ranged between 2.26 and 3.47% while *in vitro* plantlets, callus and root cultures presented values ranging from not detected up to 0.14%. The rutin content of the samples presented a similar trend with maxima between 0.99 and 3.35% for the aerial parts of the plants to 0.02 to 0.20% for plantlets, 0.12% for cell suspensions and 0.28% for callus. Rutin was not detected in the roots grown by the TIS principle. The CA and scopoletin content in the aerial parts of *F. imbricata* ranged between 0.22–1.15 and < 0.01–0.55%, respectively. In the plantlets, the concentration of CA was 0.29 to 1.48% with scopoletin in the range 0.09 to 0.64% while in the callus sample, the CA and scopoletin content were 0.46 and 0.66%, respectively. A very different result was found in roots grown by TIS, where both OA and rutin were not detected and its main secondary metabolite, scopoletin was found between a range of 0.99 and 1.41% with CA between of 0.11 and 0.42%.

Key words: Fabiana imbricata, *in vitro* Propagation, Secondary Metabolite Content

Introduction

The Andean medicinal plant *Fabiana imbricata* Ruiz et Pav. (Solanaceae) is known under the common name "pichi" or "pichi romero" and is recommended as a diuretic, digestive and to treat kidney complaints (Razmilic *et al.*, 1994). The plant displays several biological activities including diuretic effect, inhibition of the enzyme β-glucuronidase and antifeedant activity of some constituents towards the greenbug *Rhopalosiphum padi* (Schmeda-Hirschmann *et al.*, 1994, 1995).

The crude drug contains among its secondary metabolites the flavonoid rutin and the coumarin scopoletin as well as oleanolic acid and several sesquiterpenoids (Schmeda-Hirschmann and Papastergiou, 1994). The main terpenoid of the aerial parts of *F. imbricata* is oleanolic acid. Recent studies have shown that the triterpene oleanolic acid (OA) displays gastroprotective effect in rats and mice with very low toxicity (Astudillo *et al.*, 2002).

Other secondary metabolites with gastroprotective activity present in *F. imbricata* were also considered in the study. It has been reported that rutin prevented reflux oesophagitis and gastric secretion in rats by inhibiting gastric acid secretion, oxidative stress, inflammatory cytokine production, and intracellular calcium mobilization in PMNs in rats (Shin *et al.*, 2002). Chlorogenic acid is one of the major components of the polar extracts of *F. imbricata.* It is also a main product in the *n*-butanol extract of *Acanthopanax senticosus* and showed a

Abbreviations: BA, benzyladenine; GA3, gibberellic acid; IAA, indoleacetic acid; IBA, indole butyric acid; MS, Murashige & Skoog medium; NAA, naphthaleneacetic acid; 2,4-D, dichlorophenoxyacetic acid.

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significantly inhibitory effect on the stress-induced gastric ulcer (Fujikawa *et al.*, 1996). Biological activities reported for scopoletin include anti-inflammatory effect (Muschietti *et al.*, 2001), inhibition of the monoamine oxidase (Yun *et al.*, 2001) and inducible nitric oxide synthase (Kang *et al.*, 1999; Kim *et al.*, 1999), hepatoprotective effect (Kang *et al.*, 1998).

As *Fabiana imbricata* is collected from wild populations the selection of individuals with high bioactive content for cultivation purposes is advisable. However, there are no data on the seasonal variation of the secondary metabolites in the wild growing populations. Furthermore, there is no information on the secondary metabolite production of *in vitro* cultures and its variation according to explant-type and growth regulators. The aim of this work was to evaluate conditions that allow the regeneration of different organs of *F. imbricata* grown under *in vitro* conditions as an alternative for active compound production and regeneration of selected plants.

Materials and Methods

Plant material

Wild growing *Fabiana imbricata* was collected in the western Andean slopes near Altos de Chillan, Las Trancas, VIII Region, Chile, from November to July during the years 2000 and 2001. Voucher herbarium specimens have been deposited at the Herbario de la Universidad de Talca. For analysis, the aerial parts of *F. imbricata* were oven-dried at 40 °C and powdered. *In vitro* cultures and plantlets were lyophilized and homogenized in a waring blender.

In vitro propagation

All plant growth regulators, standard media and agar used for cell and tissue culture were obtained from Duchefa Biochemie BV (Haarlem, The Netherlands) or Sigma Chemical Co. (St. Louis, USA). Nodal sections of approx. 2.5 cm long, taken from plants grown in the field and maintained in the greenhouse for 1 month, were aseptically cultivated under *in vitro* conditions for induction of new shoots and callus formation. The explants were cultured in Pyrex tubes (25 mm × 130 mm) containing 12.5 ml Murashige and Skoog (1962)-liquid nutrient medium (MS) with 3% sucrose, provided with Whatman No 1 paper bridges in presence of one (2,4-D) or various growth regulators (combinations of NAA, BA and GA_3). For callus, root and plantlet formation, new shoots were subcultured in media provided with IAA, IBA or NAA. All explants were maintained in a light regime of 14 h at 48 μmol m^{-1} s^{-1} provided by daylight fluorescent lamps (Philips TLT 40W/54 R. S.), at a temperature of 22 ± 1 °C. The maintenance of roots and shoots was performed by cutting pieces of 10 mm that were cultured on MS basal salt mixture including 40 g/l sucrose and grown at 24 °C in darkness, subcultured monthly. *In vitro* regenerated plantlets were subcultured every four weeks in liquid medium devoid of plant regulators and sucrose at 24 °C with a 12 h light/darkness photoperiod.

Rapid organ multiplication

Massive propagation of shoots and roots was achieved using TIS that allows optimization of gas and nutrients exchange (Etienne and Berthouly, 2002). The device consisted of several 5 l glass bottles as cultivation vessels equipped with a layer of Whatman No 1 chromatogram paper, giving to the shoots similar conditions as the plants in glass tubes. Regenerated shoots from nodal sections were immersed in the liquid medium during 10 min every four hours. Nutrients consisted in ½ strength MS-basal medium supplemented with myo-inositol (100 mg/l), peptone (1 g/l), glycin (2 mg/l), thiamine HCl (0.1 mg/l), pyridoxine HCl (0.5 mg/l), nicotinic acid (0.5 mg/l), sucrose (20 g/l) and agar (7.5 g/l), pH 5.6 before autoclaving (medium 1). Another nutrient medium (medium 2) assayed consisted in MS-basal medium supplemented with thiamine HCl (1 mg/l), BAP (1 mg/l), GA_3 (0.5 mg/l), IAA (1 mg/l), sucrose (30 g/l), Gelrite (3.8 g/l), pH 5.8 before autoclaving.

Callus maintenance and cell suspensions

For cell proliferation (no organogenesis), approximately 3 g callus formed at nodal sections (Table I, treatment 3) were cultured in 100 ml Erlenmeyer flasks containing 20 ml MS-medium enriched with nicotinic acid (1 mg/l), pyridoxine HCl (1 mg/l), thiamin HCl (10 mg/l) including NAA (0.25 mg/l), 2,4-D (0.25 mg/l), kinetin (0.25 mg/l), sucrose (30 g/l), agar (7.5 g/l), pH 5.8 before autoclaving. Cultures were maintained in constant agitation (60 RPM) in the dark at 24 °C and subcult-

ured monthly by transferring small pieces to fresh medium. Cell suspensions were subcultured every week by inoculating the samples (8 g fresh weight) into 60 ml fresh medium.

Sample preparation and determinations

Samples of approximately 190–210 mg of dry material were extracted with dichloromethane (DCM) and MeOH, respectively, in a Soxhlet during 30 min with 90 ml of each solvent. The DCM- and MeOH-extracts were filtered and separately dried under reduced pressure. Recovery percentage was estimated by spiking an inert support with a known amount of oleanolic acid, rutin, scopoletin and chlorogenic acid. The content of the four compounds in the samples was determined by HPLC as described below. All extractions were carried out in duplicate and the quantitative determination was performed in triplicate. Results are presented as mean values ± SD.

Equipment

HPLC analyses were performed using a Merck-Hitachi (Darmstadt, Germany) equipment consisting of a L-6200 pump, a L-4000 UV detector and D-2500 chromato-integrator. Column: LiChrocart 5 μm RP 18 Select B, 250 mm.

Quantitative determination

Calibration curves were performed to estimate the oleanolic acid, scopoletin, rutin and chlorogenic acid content in the samples. The correlation between concentration/peak area was assessed by the ordinary least square regression model. The correlation coefficient r^2 was 0.99. The identity of the compounds was checked by co-injection of a reference sample isolated from *F. imbricata.* The amount of the active principles was expressed as g per 100 g of dry material. Oleanolic acid, rutin, scopoletin and chlorogenic acid were identified by their spectroscopic data, including ^{1}H and ^{13}C NMR, micromelting point and co-chromatography with standard samples.

The determination of flavonoids and phenolic acids in the samples was performed by the methodology of Häkkinen *et al.* (1998) with same modifications (Feresin *et al.*, 2002) using a 250 mm × 4 mm Lichrospher RP 18 column. The solvent system used to assess the presence of rutin, chlorogenic acid and scopoletin was as follows. Solvent A: 50 mM ammonium dihydrogen phosphate ($NH_4H_2PO_4$), pH 2.6; solvent B: 0.20 mM *ortho*-phosphoric acid (H_3PO_4), pH 2.0; solvent C: 20% solvent A in 80% acetonitrile. Rutin, chlorogenic acid and scopoletin were determined in a single run using the program previously described (Feresin *et al.*, 2002; Häkkinen *et al.*, 1998).

For the quantitative determination of oleanolic acid (Halkes, 1998), the mobile phase was: acetonitrile/water/acetic acid 70:30:0.5. Flow rate: 1.0 ml/min. Detector: UV 220 nm. Injection volumen: 20 μl. Under our working conditions, the retention time for oleanolic acid was 14.1–14.5 min.

Results and Discussion

Shoot and root regeneration, callus and cell suspension

In the present work, it was possible to establish callus, cell suspension, shoot and root cultures as well as to regenerate plantlets of *F. imbricata in vitro.* Several conditions that allowed the initiation of multiple shoot and callus and regeneration of plantlets are presented in Tables I and II. Multiple shoots were formed *de novo* starting with callus proliferation within a week in presence of NAA, BA and GA_3 or 2,4-D alone. NAA, BA and GA_3 at concentrations of 1 mg/l respectively, induced simultaneously multiple shoot formation from callus and sprouting of pre-existing axillary buds in 100% of the explants. The highest number of new shoots/explant (14.9) occurred in this after subculture in this media devoid of GA_3. Although the three initiation media also led to rhizogenesis, root formation of single shoots mostly occurred in subculture in the presence of IAA, IBA or NAA used alone or in combinations in levels of 0.25–1.0 mg/l, after one month (Table II). Under these conditions rhizogenesis occurred in 41.2–64.7% of explants after 35 days. Several roots formed simultaneously on the shoots, the highest rate was 5.6 roots per explant. Tissue browning was not observed in any of the growth conditions assayed until a period of 4 months.

Conditions presented in Table I also allowed intensive callus growth that increased five fold in biomass within 4 weeks. The established cell suspensions doubled their biomass in one week. Only when the 2,4-D concentration was raised up to 5 mg/l in subcultures, the biomass growth was inhibited (not shown).

Table I. Morphogenic responses of nodal sections and *de novo* induced new shoots of *Fabiana imbricata* 35 days and 60 days after subculture*.

Growth regulators [mg/l]	Type of explant	Culture number	Roots %	Callus %	Shoot formation %	Average shoot length [mm]	Shoots/ explant N°	Plantlets
			Treatment 1					
Initiation(1)	Nodal segments							
2,4-D 1.0		30	0.0	0.0	60.0	5.9	1.3	No
Subculture(2)	New shoots							
NAA 1.0		28	3.6	46.4	32.1	12.9	1.2	< 10%
BA 1.0								
			Treatment 2					
Initiation(1)	Nodal segments							
2,4-D 2.0		26	0.0	89.2	21.4	6.1	1.7	No
Subculture(2)	New shoots							
NAA 1.0		19	0.0	100.0	47.4	n.e.	n.e.	No
BA 1.0								
			Treatment 3					
Initiation(1)	Nodal segments							
NAA 1.0		19	0.0	100.0	100.0	5.1	4.8	No
BA 1.0								
GA_3 1.0								
Subculture(2)	New shoots							
NAA 1.0		18	0.0	0.0	26.3	n.e.	14.9	No
BA 1.0								

* Results of nodal shoots after 3 weeks, subculture of new shoots after 6 weeks.
(1) Nodal sections performed in tubes.
(2) Subculture of new shoots in 100 ml Erlenmeyer flasks.
n.e.: not evaluated.

Table II. Conditions for the induction of roots and plantlets from *de novo* induced shoots of *Fabiana imbricata* after 35 days in subculture*.

Growth regulators [mg/l]	No Cultures	Roots %	N° Roots/ explant	Remarks
Initiation				
NAA 0.3; BA 0.1; GA_3 0.01	30	0.0	0.0	only production of shoots, no callus, no plantlets.
Subcultures				
IAA 0.5	17	41.2	2.7	plantlets
IAA 1.0	19	42.1	5.6	highest% roots/explant, plantlets
NAA 0.5	20	55.0	1.7	plantlets
NAA 1.0	20	50.0	2.9	plantlets
IAA 0.25; NAA 0.25	20	45.0	2.1	plantlets
IAA 0.5; NAA 0.5	20	55.0	4.9	plantlets
IBA 0.5	17	64.7	3.2	highest% roots and plantlets
IBA 1.0	20	45.0	6.1	plantlets

* Initial cultures in tubes, explants approximately 4 weeks old , each experiment started with 20 replications.

Massive multiplication from shoot-tips formed de novo

New shoots derived from nodal sections (see above) were multiplied by branching of new axillary buds by means of the TIS. Also roots showed a very good growth in these conditions yielding a 100% increase of biomass within 3 weeks. Shoot or root inocula each of 10 g (fresh weight) allowed 20 g of harvested biomass after this period. Cultured shoots showed a high number of new shoots during a first cultivation period and were used in a second subculture for fast and massive multiplication. In the optimized systems a 10-fold increase of biomass within 8 weeks was obtained. Starting with an inoculum of 45 g fresh weight, the harvested biomass was 585 g. Shoots showed an average length of 5 cm. The condition for the biomass production of *F. imbricata* is presented in Table III.

Secondary metabolite content

The seasonal variation in oleanolic acid (OA), rutin, chlorogenic acid (CA) and scopoletin content of wild growing *Fabiana imbricata* was determined by HPLC. The content of the same metabolites was assessed in *in vitro* plantlets, callus and root cultures obtained under different growing conditions. The OA content in the aerial parts of the plant ranged between 2.26 and 3.47% while the plantlets, callus and root cultures presented values ranging from not detected up to 0.14% for the TIS-obtained roots and plantlets, respectively. The rutin content of the samples presented a sim-

Table III. Oleanolic acid (OA), rutin, chlorogenic acid (CA) and scopoletin content of wild plants, root cultures, plantlets, callus and cell suspensions of *Fabiana imbricata*. Results are presented as w/w yields in terms of dry starting material.

Sample	Wild plants			OA	Rutin	CA	Scopoletin
	collection time, month						
	november			2.61 ± 0.02	3.35 ± 0.05	0.51 ± 0.04	0.02 ± 0.01
	december			2.65 ± 0.01	3.10 ± 0.04	1.15 ± 0.03	< 0.01
	january			2.26 ± 0.03	0.99 ± 0.04	0.41 ± 0.03	0.25 ± 0.02
	february			2.44 ± 0.03	1.34 ± 0.05	0.69 ± 0.04	0.55 ± 0.01
	march			3.41 ± 0.07	1.24 ± 0.04	0.37 ± 0.02	0.14 ± 0.01
	april			3.47 ± 0.06	1.06 ± 0.03	0.22 ± 0.03	0.14 ± 0.03
	may			2.50 ± 0.20	1.58 ± 0.04	0.46 ± 0.02	0.19 ± 0.04
	In vitro cultures	Cultivation vessel	Medium*				
1	plantlet	microcontainer	medium 1	0.13 ± 0.01	0.09 ± 0.01	0.63 ± 0.03	0.09 ± 0.03
2	plantlet	RITA	medium 1 + 0.5 mg/l GA_3 + 0.1 mg/l NAA, liquid (without agar)	0.14 ± 0.01	0.20 ± 0.01	1.48 ± 0.02	0.64 ± 0.02
3	plantlet	microcontainer	medium 2, liquid (without agar)	0.06 ± 0.01	0.18 ± 0.02	0.37 ± 0.01	0.27 ± 0.01
4	plantlet	microcontainer	medium 2	0.11 ± 0.01	0.19 ± 0.01	0.29 ± 0.02	0.18 ± 0.02
5	plantlet	TIS system	2 × medium 2 (liquid), 1 × medium 1 + 0.5 mg/l GA_3 + 0.1 mg/l NAA	0.10 ± 0.03	0.02 ± 0.01	0.32 ± 0.03	0.20 ± 0.01
6	root	microcontainer	medium 1, liquid (without agar)	0.12 ± 0.04	0.03 ± 0.01	0.48 ± 0.01	0.35 ± 0.021
7	callus	microcontainer	cell culture medium	0.06 ± 0.01	0.28 ± 0.01	0.46 ± 0.01	0.66 ± 0.04
8	cell suspension	erlenmeyer flask	cell culture medium, liquid (without agar)	0.04 ± 0.01	0.12 ± 0.02	–	0.09 ± 0.01
9	bright roots	TIS system	medium 1, liquid (without agar)	–	–	0.11 ± 0.01	1.41 ± 0.01
10	bright roots with some callus	TIS system	medium 1, liquid (without agar)	–	–	0.14 ± 0.02	1.34 ± 0.05
11	dark roots with callus	TIS system	medium 1, liquid (without agar)	–	–	0.42 ± 0.01	0.99 ± 0.02
12	roots	bubble bioreactor	medium 1, liquid (without agar)	–	–	0.08 ± 0.01	1.05 ± 0.03

–: not detected.
* For the composition of medium 1 and 2 see Materials and Methods.

ilar trend, with maxima between 0.99 and 3.35% for the aerial parts of the plants to 0.02 to 0.2% for plantlets, 0.12% for cell suspensions and 0.28% for callus. Rutin was not detected in the roots grown by the TIS principle. The CA and scopoletin content in the aerial parts of *F. imbricata* ranged between 0.22–1.15 and < 0.01–0.55%. In the plantlets, the concentration of CA was 0.29 to 1.48% with scopoletin in the range 0.09 to 0.64%. In the callus sample, the CA and scopoletin content were 0.46 and 0.66%, respectively. A very different response is obtained with the roots grown by the TIS system, where both OA and rutin were not detected and its main secondary metabolite was scopoletin in the 0.99 to 1.41% range, and CA between 0.11 and 0.42%. Results are presented in Table III.

In the wild growing plants, large seasonal variations in the secondary metabolites content was observed. The highest oleanolic acid content (3.41–3.47%) was found in the plants harvested late summer (March–April). The rutin, chlorogenic acid and scopoletin content in the same plants ranged between 0.99 and 3.35, 0.22 and 1.15 and < 0.01 and 0.55%, respectively. The highest values for rutin (3.10–3.35%) were found in plants collected in November and December while the maxima for chlorogenic acid was 1.15% for the December sample and scopoletin reached its maximum on February with 0.55%.

The plantlets were characterized by low oleanolic acid (0.06–0.14%), rutin (0.02–0.20%), scopoletin (0.09–0.27%) and chlorogenic acid content, excepting one sample with higher chlorogenic acid (1.48%) and scopoletin production (0.64%).

In the biomass produced under the temporary immersion system conditions, both oleanolic acid and rutin were not detected. While the bright, beige roots presented high scopoletin content (1.34–1.41%) and chlorogenic acid ranging from 0.11 up to 0.14%, the dark brown roots presented higher concentration of chlorogenic acid (0.42%) with less scopoletin (0.99%). The roots produced in a bubble bioreactor showed a very low chlorogenic acid content with scopoletin (1.05%) as their main secondary metabolite.

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