Secondary Metabolite Content in Rhizomes, Callus Cultures and *in vitro* Regenerated Plantlets of *Solidago chilensis*

Guillermo Schmeda-Hirschmann[a,*], Miguel Jordan[b], André Gerth[c], and Dirk Wilken[c]

[a] Universidad de Talca, Instituto de Química de Recursos Naturales, Laboratorio de Productos Naturales, Casilla 747, Talca, Chile. Fax: +56 71 200448. E-mail: schmeda@utalca.cl
[b] Pontificia Universidad Católica de Chile, Facultad de Ciencias Biológicas, Departamento de Ecología, Alameda 340, Santiago, Chile
[c] BioPlanta GmbH, Deutscher Platz 5, D-04103 Leipzig, Germany

* Author for correspondence and reprint requests

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An *in vitro* culture system leading to the formation of callus and plant regeneration, starting from nodal sections and shoot tips, was developed for *Solidago chilensis* (Asteraceae). The content of the gastroprotective diterpene solidagenone as well as the phenolics chlorogenic acid (CA) and rutin was determined either in rhizomes from wild growing plants and in callus and in *in vitro* regenerated plantlets by analytical HPLC. Additionally, total phenolic and flavonoid content was assessed in plant samples, callus and cell suspensions. In terms of dry starting material, the percentual solidagenone content in nine *S. chilensis* samples ranged from 0.5–3.5% for rhizomes from wild growing plants, 0.1–0.3% for callus and 0.3% for an *in vitro* regenerated plantlet, respectively. The highest solidagenone contents were found in the wild plant during the late summer in the months of March and April (3.5–2.2%) while highest values for chlorogenic acid (0.5%) and rutin (0.4%) were detected in May, before senescence. The callus tissue and cell suspensions contained some 1.8–2.0 and 1.2% of total phenolics, respectively. CA was the main phenolic in the cell suspension while only traces were found in the callus. Rutin was not detected in the callus nor cell culture.

Key words: In vitro Propagation, Secondary Metabolite Content, *Solidago chilensis*

Introduction

The labdane diterpene solidagenone (Fig. 1) as well as its semisynthesis and biotransformation derivatives have been shown to display several biological activities, namely gastroprotection (Rodríguez *et al.*, 2002; Schmeda-Hirschmann *et al.*, 2002), insect antifeedant (Cifuentes *et al.*, 1999), and an inhibitory effect of the glucocorticoid-mediated signal transduction (Razmilic and Schmeda-Hirschmann, 2000). Solidagenone, which is not commercially available, can be isolated from the rhizomes of *Solidago chilensis* (Asteraceae) when the herb grows during late spring and summer in the Southern hemisphere (Schmeda-Hirschmann, 1988). Other secondary metabolites with gastroprotective activity present in *S. chilensis* include the flavonoid rutin and the phenolic chlorogenic acid (CA). It has been reported that rutin prevents reflux oesophagitis and gastric secretion in rats by inhibiting gastric acid secretion, oxidative stress, inflammatory cytokine production, and intracellular calcium mobilization in polymorphonucleocytes in rats (Shin *et al.*, 2002). Chlorogenic acid is one of the components of the polar extracts of *S. chilensis*. It is also a main product in the extract of *Acanthopanax senticosus* and showed a significant inhibitory effect on the stress-induced gastric ulcer (Fujikawa *et al.*, 1996).

Solidago species are used as medicinal plants at several places of the world. In a review of crude drugs used for treating urinary tract diseases, Yarnell (2002) includes *Solidago* spp. (goldenrod) among the diuretic plants while the anti-inflammatory, spasmolytic and diuretic effect of a commercially available *Solidago gigantea* extract was reported by Leuschner (1995). The rhizomes of *Solidago chilensis* are used in Paraguayan traditional medicine as a diuretic and appetizer (Schmeda-Hirschmann, 1988).

The production of the secondary metabolite *in vitro* could be an alternative for a continuous sup-

Abbreviations: BA, 6-benzyladenine; GA3, gibberellic acid; MS, Murashige & Skoog medium; NAA, naphthaleneacetic acid.

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ply of the active compounds from *S. chilensis*. The aim of this work was to develop a methodology for callus production and for micropropagation of this species and to assess the solidagenone, rutin and CA content in wild plants, callus, cell suspensions and plantlets by analytical HPLC. The *in vitro* regeneration, as well as growth conditions for callus production on this species has not been reported so far.

Materials and Methods

Plant material

The samples of wild growing *S. chilensis* were collected in Chile at Las Trancas, Altos de Chillán, VIII Region, in the western Andean slopes, 700 m above sea level from February 2000 to April 2001. One sample was collected at the road Lonquimay-Tunel Las Raices, IX Region on February 2001. Voucher herbarium specimens have been deposited at the Herbario de la Universidad de Talca.

Callus culture and in vitro propagation

Plant growth regulators, standard media and agar used for tissue culture of *S. chilensis* cells and organs were obtained from Duchefa Biochemie BV, Haarlem, The Netherlands or Sigma Chemical Co., St. Louis, USA. Leaf sections of approx. 1 cm^2, nodal sections and shoot tips of about 1 cm length were excised from plants collected from the field and maintained in the greenhouse for 1 month. Explants were disinfected with Captan (Micro Flo Company LLC, Memphis, USA) and Benlate® (Du Pont & Co. Inc., Nemours, France) (0.02% each) for 30 min under constant agitation, then washed in sterile distilled water and aseptically cultured under *in vitro* conditions. The explants were first cultured in Pyrex tubes (25 × 130 mm) containing 13 ml MS-medium (Murashige and Skoog, 1962) provided with Whatman Nr 1 paper bridges in the presence of various growth regulators like NAA, BA, and GA_3 including 3% sucrose. After callus initiation (40 d), subcultures were performed to promote callus biomass by transferring small callus pieces of approx. 100 mg to 13 ml fresh MS-medium also in combination with various levels of growth regulators and 100 mg/l glutamine. A second subculture was performed similarly. The addition of glutamine in both phases favored growth as was found in a previous work (not shown). For the preparation of cell suspensions, callus pieces were cultured under constant agitation [120 strokes/min Eberbach reciprocal shaker (Eberbach Labtools, Ann Arbor, Michigan, USA)] for one week, and later for culture and cell proliferation in flasks on an orbital shaker (60 rpm; Lab-Line, IL, USA) for other six weeks. All explants were maintained under 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 22 °C ± 1 °C.

Sample preparation and determinations

The rhizomes of *Solidago chilensis* were ovendried at 40 °C and ground in an electric mill. Callus cultures and *in vitro* plantlets were lyophilized and homogenized in a Waring blender. Some 190–210 mg of dry material was extracted with methanol in a Soxhlet extractor during 30 min with 150 ml MeOH. The organic phase was filtered and processed to dryness under reduced pressure.

HPLC analysis

A Merck-Hitachi (Darmstadt, Germany) HPLC equipment with an UV detector (Model Series L-4000) and a data processor were used. Column: Superspher® 60 5 μm RP 18 Select B, 250 mm, particle size 5 μm.

Quantitative determination

A representative sample of each extract was dissolved in HPLC-grade MeOH, to a final concentration of 1.0 mg/ml. Calibration curves were performed to estimate the solidagenone, rutin and chlorogenic acid content in the samples plotting the area of the standard vs. concentration. The correlation between the 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 reference samples isolated from *Solidago chilensis*. The amount of the active principles was expressed as g per 100 g of dry material. Solidagenone, rutin and chlorogenic acid were identified by their spectroscopic data, including ^1H and ^{13}C NMR, micromelting point and co-chromatography with standard samples.

The determination of rutin and chlorogenic acid in the samples was performed as reported by Feresin *et al.* (2002). The solvent system used to assess the presence of rutin and chlorogenic acid was as follows: Solvent A: 50 mM ammonium dihydrogen phosphate, pH 2.6; solvent B: 0.20 mM *ortho*-phos-

phoric acid, pH 2.0; solvent C: 20% solvent A in 80% acetonitrile. Rutin and chlorogenic acid were determined in a single run using the program previously described (Feresin *et al.*, 2002). The solidagenone content was determined under the following experimental conditions: Detection: UV, 220 nm; mobile phase: MeOH/H_2O (70:30 v/v); flow rate: 0.9 ml/min. In our working conditions, the retention time (Rt) of solidagenone was 12 ± 1 min. Recovery percentage was estimated by spiking an inert support with a known amount of solidagenone (10–15 mg). Percentual recovery of solidagenone was 91.6%. Measurements were carried out in triplicate and results are presented as mean values ± SD.

Results and Discussion

The aim of all *in vitro* culture experiments was to establish cell and callus proliferation of *S. chilensis* to assess the solidagenone (Fig. 1), CA and rutin production in comparison with contents in wild growing plants. During the present work, conditions for callus growth and plantlet regeneration for this species were also determined; however assays were not conducted to assess plantlet formation but mainly to promote callus proliferation for solidagenone production. Results are summarized in Table I. Shoot tips and nodal segments but not leaf explants formed callus within 35 d: in various treatments the tips and axillary buds reassumed growth and formed roots, leading to platelets, as indicated below. Callus formation was intense and observed in all treatments, *i.e.* in 100% of shoot tips and 75% of the nodal sections. Leaf explants did not form calli. Generally, the ground callus of different origin had a friable green appearance after 30 d turning gray or brown after 60 d when not subcultured. On the contrary, the callus derived from shoot tips in continuous subculture showed green spots on the surface after 60 d but histological examination showed that these structures did not correspond to new shoots or somatic embryoids (not shown). In subcultures, a three- to eight-fold increase of callus biomass was observed within 4 weeks in the presence of various levels of NAA and BA supplemented with 100 mg/l glutamine but not when used as single regulators (Table I). Root formation was observed in a low frequency in some nodal explants, leading to plantlets in the presence of 1.0 mg/l of NAA, BA and GA_3, respectively, that were used for solidagenone analysis.

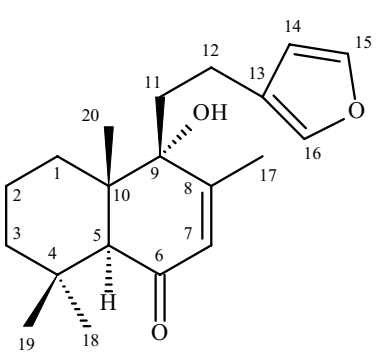

Fig. 1. Structure of solidagenone.

The solidagenone, CA and rutin content of nine *S. chilensis* rhizome collections, three callus cultures and rooted nodal sections were analyzed by HPLC. Results are summarized in Table II. The solidagenone content in terms of dry starting material was 0.5–3.5% for the rhizomes, 0.1–0.3% for callus and 0.3% in *in vitro* plantlets. The highest solidagenone content of rhizomes in wild growing plants was observed for late summer, in the months of March and April, both corresponding to the year 2000 and 2001 while the highest CA (0.5%) and rutin (0.4%) content were observed in May before senescence. The callus culture and cell suspension contained some 1.8–2.0% and 1.2% of total phenolics, respectively. CA was the main phenolic in the cell suspension while only traces were found in the callus culture. Rutin was not detected in the callus nor cell culture.

This results make possible, in some extent, to multiply selected material from the field. A more accurate determination of the maximal solidagenone content in wild plants should consider genetic variation found in additional populations of different stands, soil condition and nutrition and increasing sampling in at least a weekly basis. The potential of plant cell cultures in the search for new active compounds has been outlined by Schripsema *et al.* (1996) and Stafford *et al.* (1999). In *S. chilensis* as for many other species described in the literature, cell cultures generally synthesize low amounts of phytochemicals. Although many compounds do not achieve high concentrations in undifferenciated cell cultures plant cells can produce novel compounds which can be or not related with those previously isolated in the entire plant (Stafford *et al.*, 1999). Another approach in future work is to establish tissue and organ cultures of

Table I. Best growth responses found in different explant types of *Solidago chilensis* cultured *in vitro*.

Type of explant	Growth regulators [mg/l]	Culture number	Callus (%)	Root (%)	Roots/ explant number	Remarks
Leaf sections	First Culture					
	NAA 0.01	30	0	0	0	No response
	BA 1.0					
	GA_3 0.1					
Nodal sections						
NS 1	NAA 1.0	60	75	5	1	Green callus. A few rooted plantlets (n = 3)
	BA 1.0					
	GA_3 1.0					
Shoot tips						
ST 1	NAA 1.0	20	100.0	0	0	Callus initiation only
	BA 1.0					
ST 2	NAA 1.0	20	100.0	0	0	Callus initiation, intensive growth, with green spots
	TDZ 0.5					

Callus	1st Subculture[a]	2nd Subculture	Callus weight[b] [mg]	Root (%)	Roots/ explant number	Remarks
CT 1	NAA 1.0	NAA 0.1	302.5	0	0	
	BA 1.0	BA 0.1				
CT 2	NAA 1.0	NAA 0.1	891.0	0	0	Most intensive growth
	BA 1.0	BA 1.0				
CT 3	NAA 1.0	NAA 1.0	455.0	0	0	
	BA 1.0	BA 1.0				
CT 4	NAA 1.0	NAA 1.0	363.8	0	0	Less callus growth
	BA 0.5	BA 1.0				

[a] Callus cultures derived from shoot tips grown in the presence of 1.0 mg/l NAA and 0.5 mg/l BA. 1st and 2nd subculture media were supplemented with 100 mg/l glutamine.
[b] Fresh weight after 60 d after 10 explants/treatment. NS, nodal sections; ST, shoot tips; CT, callus treatment.

Solidago spp. for production of secondary metabolites co-cultured with strains of *Agrobacterium, i.e.* Inoguchi *et al.* (2003) indicated the production of polyacetylenic compounds in roots of *S. altissima* infected with *Agrobacterium rhizogenes*.

Conclusions

Conditions for rapid *in vitro* cell and callus proliferation as well as plantlet formation were established for *Solidago chilensis* (Asteraceae) starting from nodal sections and shoot tips. The rate of callus growth depended on NAA/BA levels as well as root formation, but the latter only took place on callus derived from nodal sections. An HPLC method was developed for rapid quantification of solidagenone, CA and rutin in rhizomes, callus and plantlets of *S. chilensis*. The CA content of the rhizomes was higher in most samples collected in late summer (0.3–0.5%) but cell suspensions showed a much higher level (1%). On the contrary, the rutin content was quite low compared with the Chilean crude drug *Fabiana imbricata* (Schmeda-Hirschmann *et al.*, 2004). Despite the 5-fold variations of solidagenone observed in calli, compared to wild plants, its content was relatively low. However, calli that evidenced less growth produced higher solidagenone contents compared to active proliferating calli (Table II). It is assumed that this effect is related with the lack of cellular differentiation although solidagenone synthesis was increased according to the growth regulators (Dougall, 1980). Summarizing, besides the high amounts of callus biomass produced and the synthesis of some natural compounds, the assays led to the regeneration of a few rooted plants from nodal explants, that were successfully transferred to the soil.

A comparison of the phenolic compounds in two *Solidago* species from *in vitro* culture showed that regenerated plants contained phenolic glyco-

Table II. Solidagenone, total phenolic and flavonoids, chlorogenic acid and rutin content (%) in *Solidago chilensis* rhizomes, callus and plantlets.

Sample and Data	Solidagenone content[a]	Total phenolics as gallic acid	Total phenolics as tannic acid	Total flavonoids	Chlorogenic acid	Rutin
Wild growing plants						
LT, 18.02.2000	0.65 ± 0.05	0.73	0.86	0.27	0.27 ± 0.02	0.17± 0.01
LT, 18.02.2000	0.52 ± 0.06	0.86	0.76	0.26	0.19 ± 0.01	0.14 ± 0.02
L, 10.02.2001	0.99 ± 0.06	0.38	0.36	0.15	0.14 ± 0.02	0.10 ± 0.01
LT, 22.02.2001	1.39 ± 0.02	0.75	0.77	0.23	0.18 ± 0.03	0.03 ± 0.01
LT, 20.03.2001	3.48 ± 0.25	0.99	0.91	0.30	0.31 ± 0.02	0.34 ± 0.03
LT, 14.04.2001	2.24 ± 0.08	1.10	1.08	0.31	0.31 ± 0.03	0.07 ± 0.01
LT, 22.04.2000	1.41 ± 0.12	0.96	0.95	0.43	0.05 ± 0.01	0.17 ± 0.02
LT, 29.04.2001	0.59 ± 0.04	1.07	1.03	0.28	0.36 ± 0.03	0.07 ± 0.01
LT, 20.05.2000	1.18 ± 0.08	1.06	0.96	0.37	0.46 ± 0.05	0.37 ± 0.05
Callus forming directly on a nodal section in the presence of 1 mg/l NAA, BA and GA_3, respectively	0.09 ± 0.01 (NS 1)	–	–	–	nd	nd
Callus from 2nd subculture in 0.1 mg/l NAA and 1.0 mg/l BA	0.06 ± 0.01 (CT 2)	–	–	–	nd	nd
Callus from 2nd subculture in 1.0 mg/l NAA and 1.0 mg/l BA	0.25 ± 0.05 (CT 4)	–	–	–	nd	nd
Callus 1037 BioPlanta		1.97	1.83	0.55	0.009 ± 0.05	
In vitro plantlets						
Plantlets derived from a rooted nodal section grown in 1 mg/l NAA, BA and GA3, respectively	0.28 ± 0.08 (NS 1)	–	–	–	nd	nd
Cell suspension 1040 BioPlanta		1.20	1.21	0.36	1.00 ± 0.05	nd

[a] Data are presented as w/w yields in terms of dry material.
Abbreviations: CT 2 and CT 4 are treatments for callus, NS 1, callus and plantlets from nodal sections, presented in Table I. Collection data: day, month, year. LT: Las Trancas; L: Lonquimay; C: callus culture; – : not determined; nd: not detected.

sides and flavonoids with a similar pattern to that observed in the naturally growing plants. The callus tissue, however, contained mainly caffeoylquinic acid and only traces of flavonoids (Thiem *et al.*, 2001).

Further research should deal with the increase of solidagenone content as well as with the composition of other secondary metabolites in this species.

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