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DISTRIBUTION OF CALCITRIOL ACTIVITY IN *SOLANUM GLAUCOPHYLLUM* PLANTS AND CELL CULTURES

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Key Word Index—*Solanum glaucophyllum*; Solanaceae; steroidal glycosides; cholecalciferol; calcitriol; plant cell cultures; CaBP bioassays.

Abstract—Distribution studies showed for the first time that calcitriol activity is present not only in leaves, but also in berries, stems and roots of *Solanum glaucophyllum* plants, in both aglycone and glycoside form. Berries contained the highest concentration of active derivatives and leaves the lowest. Substantial activity was also demonstrated for the plant cell cultures. The implications of these findings for plant breeding and yield optimization are discussed.

INTRODUCTION

Solanum glaucophyllum Desf. is a calcinogenic plant growing wild in South America, where it causes the intoxication of grazing animals [1]. Early work demonstrated that glycoside conjugates of calcitriol [**1c** 1α,25-dihydroxycholecalciferol, 1α,25-$(OH)_2D_3$] occur in minute concentrations in the plant leaves [2]. Further investigation revealed the presence of three fructoglucosides of 1α,25-$(OH)_2D_3$ [3], and also of the vitamin D_3(**1a**, cholecalciferol) and 25-OH D_3 (**1b**) in the aglycone portion of the glycosides [4], thus suggesting that different vitamin D_3 sterols might be conjugated to various carbohydrate moieties or sequences, affording a series of naturally occurring calcinogenic glycosides [5]. The pharmaceutically useful hormone 1α,25-$(OH)_2D_3$ itself is a *seco*-sterol important in regulating mineral metabolism and bone resorption in humans and animals [6].

The calcinogenic activity of *S. glaucophyllum* was earlier reported to reside chiefly in leaves and apparently none was found in stems [7, 8]. Also the calcinogenic activity predominated in the vegetative rather than in the reproductive development stage [9]. The active principles of the leaves are soluble in polar solvents and, reportedly, not extracted with nonpolar solvents, notwithstanding some conflicting results suggesting activity in ethereal plant extracts [10, 11]. However, the possible presence of the free aglycone in some particular plant batches had been suspected at the time [12], because of the interaction of unhydrolysed plant extracts with an intestinal cytosolic receptor [13] held to interact only with free 1α,25-$(OH)_2D_3$ [14]. These controversial reports prompted us to investigate the distribution of calcinogenic glycosides and/or aglycones in various tissues of *S. glaucophyllum* plants. We wish to report herein the results of a study on the distribution of 1α,25-$(OH)_2D_3$ activity in different organs and cell suspensions of this species.

RESULTS AND DISCUSSION

The exhaustive extraction of the different parts of *S. glaucophyllum*, first with a nonpolar solvent (methylene chloride), followed by subsequent extraction of the remaining material with a polar solvent (80% aqueous ethanol), allowed a selective separation of aglycones from glycosides. In fact, the latter derivatives have estimated M_r of 1000–2000 [3, 13] and should be virtually insoluble in nonpolar solvents. Conversely, had the successive order of extraction solvents been reversed, i.e. the polar solvent used first, some aglycone material could have been solubilized by ethanol and removed into the glycoside fraction.

Under these conditions, the berries yielded the highest total amount of extractable material (i.e. methylene chloride extracts and/or ethanol extracts, *ca* 30% of the dried sample weight), followed by leaves (*ca* 20%). For stems and roots, most of the total extractable material (*ca* 10%) was present in the ethanol fractions (Table 1).

The feeding of a high-strontium, low-calcium, vitamin D-replete diet to chicks was previously found to depress the formation of intestinal calbindin-D_{28K}(CaBP) [15] due to the inhibition of the renal formation of 1α,25-$(OH)_2D_3$ from 25-OH D_3 by this diet [16]. Administration of a source of 1α,25-$(OH)_2D_3$ to strontium-inhibited chicks restores the capacity of the intestine to synthesize CaBP, and this thereby serves as an indicator of the presence of a 1α,25-$(OH)_2D_3$-like derivative in the test sample. The ability of extracts of the various plant parts and of the cell suspensions of *S. glaucophyllum* to stimulate the synthesis of CaBP in strontium-inhibited chicks provides definitive evidence for the presence of compounds with 1α,25-$(OH)_2D_3$-like activity therein.

The highest concentration of the active principle, as indicated by the induction of the synthesis of CaBP, is found in the berries as glycosides, and their value is

Table 1. Distribution of calcitriol activity* in different parts of *Solanum glaucophyllum*

Plant part†	Contribution to total dry plant weight	Crude CH_2Cl_2 extract (aglycones)‡			Crude EtOH extract (glycosides)§			Ratio of glycosides/ aglycones extracts		Total extract (aglycones and glycosides) CaBP	
		Yield		CaBP‖ (μg/mg protein)	Yield		CaBP‖ (μg/mg protein)			(μg/mg protein)	(%)
	(%)	(mg)	(%)		(mg)	(%)		Weight	CaBP		
Leaves	18.1	172	8.6	0.2±0.2	269	13.4	2.5±0.8	1.6	12.50	2.7	11.1
Berries	3.9	262	13.1	4.0±1.4	372	18.6	9.0±2.0	1.4	2.25	13.0	53.5
Stems	44.8	14	0.7	3.2±1.1	252	12.6	1.2±0.5	18.0	0.38	4.4	18.1
Roots	33.2	11	0.5	2.2±0.8	215	10.7	2.0±0.8	19.5	0.91	4.2	17.3

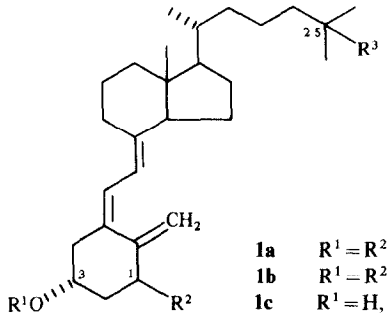
*Expressed as concentration of duodenal CaBP (calbindin-D_{28K}).

†The mean ±s.e. of 4 samples was 18.2±1.0 μg CaBP/mg protein for the calcitriol-treated group and 0.61±0.10 μg CaBP/mg protein for the propylene glycol control.

‡Originating from 2 g dry plant material.

§Originating from 2 g dry plant material previously extracted with CH_2Cl_2.

‖Values represent the mean ±s.e. of 8 samples.



1a $R^1=R^2=R^3=H$
1b $R^1=R^2=H$, $R^3=OH$
1c $R^1=H$, $R^2=R^3=OH$

significantly greater ($p<0.05$) than that of glycosides in leaves, stems and roots (Table 1). The leaves have the lowest content of aglycones of the various fractions and significantly ($p<0.05$) less than the parent glycosides level and than the aglycones in berries, whereas all other plant organs contain substantial amounts of the aglycone form of the active substance. Significant amounts of the glycoside derivatives of 1α,25-$(OH)_2D_3$ are present in each of the plant parts, and most of the 1α,25-$(OH)_2D_3$-like material in leaves and berries is in the form of the glycoside. In stems the reverse distribution is apparent and, in roots, the amounts of aglycones and glycosides are about the same. The total activity (aglycones and glycosides) in stems and roots is higher than that found in the leaves.

In previous studies, only the leaves of *S. glaucophyllum* were assayed [14, 17] and it seems indeed of interest to report that 1α,25-$(OH)_2D_3$-like activity is also present in all other parts of the plant in comparable or even higher amounts. Noteworthy, too, is the ratio of glycosides to aglycones in the various plant parts, the variability (0.38 and 12.50 for stems and leaves, respectively, see Table 1) probably reflecting differences in the metabolism and accumulation of these derivatives in each organ. Thus, the exceedingly high ratio of glycosides to aglycones in leaves, which reportedly are a site of hydroxylation of the aglycones **1a** and **1b** [4], might suggest that the glycosylation of the aglycones occurs in leaves in the presence of the appropriate glycosylating enzymes and/or glycosyl transferases; the resultant glycosides are subsequently translocated to the other organs for storage, the berries being apparently a preferred site of accumulation.

The study of *S. glaucophyllum* in tissue culture has, so far, undergone little investigation. However, seeds of this plant species and callus tissue cultures were shown to produce steroids (sitosterol and diosgenin) and a steroidal alkaloid (solasodine) [18]. In order to find out whether the *in vitro* synthesis of the active principle can take place without exposure to daylight, we performed preliminary experiments with *S. glaucophyllum* cell cultures in the dark. Our results indeed point to appreciable activity formed under these conditions. Two of the cell suspensions of *S. glaucophyllum* synthesized significant amounts of the active principle *in vitro* (Table 2, entries 2 and 4, the former significantly greater than the latter; see also [19]). The wide variability of 1α,25-$(OH)_2D_3$ activity among the different cell suspensions might reflect genotypic differences between hypocotyls from which the cell cultures originated, and can be used to develop high-

Table 2. Calcitriol activity* of cell suspensions from *Solanum glaucophyllum*

Cell suspension no.	Cells dry weight (g/flask)	CaBP† (μg/mg protein)
1	0.362	0.4±0.3
2	0.306	9.3±0.8‡
3	0.424	1.5±0.3
4	0.473	2.8±1.4
5	0.410	1.5±0.3

*Expressed as concentration of duodenal CaBP (calbindin-D_{28K}).

†Values represent mean ±s.e. of 4 samples per cell suspension. The calcitriol control values were 18.2±1.0 μg/mg protein. The propylene glycol control values were 0.61±0.10 μg/mg protein.

‡Significantly greater than cell suspensions 1, 3, 4 and 5, at $p<0.05$.

yielding cell clones. We have also observed a comparable variability in activity at the plant level.

The activity present in one of the cell cultures incubated in the dark (Table 2, entry 2) is higher than that of the leaves, stems and roots of the plant, and comparable to that of the berries. It is certainly possible that exposure of the cell cultures to daylight or ultraviolet irradiation, and optimization of the culture conditions would enhance activity. At any rate, the formation of 1α,25-$(OH)_2D_3$-like derivatives by cell suspensions of *S. glaucophyllum* provides a potentially useful technology for an economically feasible biosynthetic approach.

Our findings on activity distribution within the plant might prove important for developing the appropriate cultivation practices needed to maximize the yield. In fact, although the berries possess the highest activity, their contribution to the total dry biomass yield is only 3.9% (see Table 1). The low berry yield of the species is a combined result of low flowering rate and fruit set on the inflorescences (0–40 berries per plant), and low dry weight of the berries (0.1–0.3 g per berry). In contrast, the stems and roots contribute substantially to the total biomass yield and plant activity (Table 1) and, in addition, are easier to process than the leaf material which is contaminated with pigments. Especially attractive seems to be the use of stems and roots for the extraction of aglycones since small amounts of total extractable materials were found to display high activity (Table 1). Production conditions should therefore be directed towards the exploitation of the total biomass of *S. glaucophyllum* plants rather than their leaves alone, as done currently (the dry leaf yield accounts for *ca* 3.3 tons per hectare at two seasonal harvests). Our data also point out that the content of 1α,25$(OH)_2D_3$-like derivatives in *S. glaucophyllum* is likely to be much higher than previously reported.

EXPERIMENTAL

Plant material and growth conditions. Seeds of *S. glaucophyllum* harvested on various accessions originating in Argentina were bulked and sown on beds 1.8 m wide at the Experimental Station at Bet Dagan. Plant spacings were 20 and 15 cm, between and within rows, respectively. After 6 months of growth, plants from four randomized plots (7.2 m^2 each) were dug out at a depth of 25–30 cm. The different parts of the plants (leaves, stems, fruits and roots) were separated, oven-dried at 60° for 1 week, weighed and then extracted.

Cell cultures of S. glaucophyllum. Seeds of *S. glaucophyllum* were surface-sterilized with commercial 20% Chlorox soln and germinated on 1% agar at 28° in dark. For callus induction, hypocotyls from young seedlings (2-weeks-old) were transferred to petri dishes containing Musashige and Skoog medium [20] supplemented with 1 mg/ml 2,4-D (2,4-dichlorophenoxy-acetic acid), 0.3 mg/ml kinetin, and solidified with 0.8% agar. Following 2 weeks' incubation in the dark at 25°, friable white callus formed on the hypocotyls. Suspension cultures were initiated by dispersing small fragments of the callus in 100 ml flasks filled with 20 ml of medium without agar, and placed in the dark on a gyratory shaker (100 rpm) at 25°. Subculturing was done every 3 weeks by placing 10 ml cells into 250 ml Delong flasks filled with 50 ml of the medium solution. The cells from five different suspensions, each originating from a different hypocotyl, were collected by filtration 18 days after inoculation, lyophilized, and bioassayed for 1α,25-$(OH)_2D_3$ activity.

Extraction of S. glaucophyllum *organs. Free sterols.* Samples (2 g) of plant organs were refluxed with CH_2Cl_2 (50 ml) for 6 hr, and then left 48 hr at room temp. The suspensions were filtered and the filtrates were evapd to dryness under red. pres, and subsequently bioassayed for activity. *Glycosides.* CH_2Cl_2-extracted plant materials obtained as described above were further extracted with 80% aq. EtOH (50 ml) under reflux for 6 hr, and then left overnight at room temp. Following filtration of suspensions, the filtrates were concentrated under red. pres. to remove most of the alcohol. Residual water was removed by lyophilization and the dried extracts subjected to bioassay for activity.

Determination of 1α,25-$(OH)_2D_3$*-like activity.* The different preparations derived from *S. glaucophyllum* were solubilized in 15 ml H_2O or propylene glycol. Those preparations that were relatively insoluble in either solvent were suspended as fine particles by homogenization with a polytron (Brinkmann Instruments, Inc., Westbury, NY; 30 sec at setting no. 6). One-day-old White Leghorn cockerels (Halls Bros., Wallingford, CT) were fed a commercial chick starter diet (Agway Inc., Ithaca, NY) for 2.5 weeks, at which time the chicks were fed a high-strontium diet. This semi-synthetic diet (Teklad Inc., Madison, WI) contained 0.1% Ca, 4.4% $SrCO_3$ and 1200 i.u. vitamin D_3 per kg diet, and all other nutrients at recommended levels. After one week on the high-strontium diet, the chicks were sub-divided into groups of four and given *per os* 1 ml of the plant extracts on three successive days. On the fourth day, the chicks were killed, the duodenum recovered, and the CaBP content of the duodenal mucosa was determined by a radial immunodiffusion assay, as described previously [21]. Protein concentrations were determined by the procedure of Lowry *et al.* [22], using bovine serum albumin as the reference protein. The negative control group (4 chicks) was given propylene glycol *per os* on the same schedule as the treated groups. The positive control group (4 chicks) was given 0.1 μg 1α,25$(OH)_2D_3$ in propylene glycol *per os* on the same schedule. The CaBP data, from which the negative control data had been subtracted, are expressed as the mean ± s.e. of the replicate samples assayed.

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