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Nicotiana glauca: another plant species containing vitamin D_3 metabolites

Mario Skliar, Alejandro Curino, Lorena Milanesi, Silvia Benassati, Ricardo Boland *

Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670, (8000) Bahía Blanca, Argentina

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Abstract

Vitamin D_3-related compounds have been detected in various plant species, mostly belonging to the Solanaceae. In this work we show that *Nicotiana glauca*, a widespread member of this taxonomic family, contains 7-dehydrocholesterol, vitamin D_3 and hydroxylated derivatives bearing precursor-product metabolic relationships in vertebrates. Leaves collected in the field and callus cultures were used. By means of specific radioreceptor binding assays and mass spectrometry of purified fractions obtained from plant tissue lipid extracts by Sephadex LH-20 and Sep-Pak C18 chromatography followed by HPLC, we established the presence of 7-dehydrocholesterol, vitamin D_3, 25(OH)-vitamin D_3 and 1α,25$(OH)_2$-vitamin D_3 (1α,25$(OH)_2D_3$), the latter being a hormonally relevant metabolite in animals. These results indicate that *N. glauca* may represent a useful species in which to characterize the biosynthetic pathway and physiological functions of vitamin D_3 compounds in plants. In addition, tissue culture of *N. glauca* could become a significant tool for biotechnological production of 1α,25$(OH)_2D_3$.

Keywords: *Nicotiana glauca*; Solanaceae; 7-Dehydrocholesterol; Vitamin D_3; Vitamin D_3 metabolites; 1α,25$(OH)_2$-vitamin D_3; Mass spectrometry

1. Introduction

The occurrence of calcinosis in grazing animals in various parts of the world [1,2] led to the isolation and identification in plants of vitamin D_3-related compounds [3–5], thought previously to be generated only in vertebrates during the metabolic conversion of 7-dehydrocholesterol to the pluripotent hormone 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$). These intermediates (7-dehydrocholesterol, vitamin D_3, 25$(OH)D_3$, 1α,25$(OH)_2D_3$) may be found in plants both as aglycones and glycoside derivatives. Various investigations employing in vitro systems have shown that vitamin D sterols affect root growth and differentiation [6–8] through a stimulation of root calcium uptake and calmodulin synthesis [9–11], suggesting a physiological function for these compounds.

Although several species which have been shown to contain vitamin D_3 metabolites are included in different taxonomic families, most of them belong to the Solanaceae [4,12–15]. In the present work we have investigated the presence of these compounds in *Nicotiana glauca* Graham (Solanaceae; n.v. Tree tobacco), a world-wide member of this family originating in South America (Palan palan or Tabaco moro) [16,17]. By means of specific radioreceptor binding assays, HPLC and mass spectrometry we demonstrate that this plant species contains 7-dehydrocholesterol, vitamin D_3 and its hydroxylated metabolites.

* Corresponding author. Fax: +54-291-4595130.

E-mail addresses: mskliar@criba.edu.ar (M. Skliar)., rboland@criba.edu.ar (R. Boland).

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2. Materials and methods

2.1. Materials

Solvents used for isolation and chromatographic procedures were HPLC grade (J.T. Baker, Phillipsburg, NJ, USA). Compounds for culture media, 7-dehydrocholesterol and Sephadex LH-20 were obtained from Sigma-Aldrich (St Louis, MO, USA) and Gibco BRL (Gaithersburg, MD, USA). The Sep-Pak cartridges were from Waters (Milford, MA, USA). The LiChroCART cartridge for HPLC analysis was from Merck (Darmstadt, Germany). Other reagents were of analytical grade. Vitamin D_3, 25(OH)-vitamin D_3 and $1\alpha,25(OH)_2D_3$ were a gift from Dr Lise Binderup, Leo Pharmaceutical Products (Ballerup, Denmark).

2.2. Cultures

To culture *N. glauca* tissue (callus), aseptic leaf explants from plants collected in Bahía Blanca, Buenos Aires Province, Argentina, were inoculated in solidified (0.8% agar) Murashige-Skoog [18] medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 0.5 mg/l) and kinetin (0.2 mg/l). The tissue was grown at 25°C and during the culture period it was not illuminated. The calli started to develop after ~15 days. Subculture was performed when the entire surface of culture flasks was covered with tissue (~2 months). Compounds were isolated after the first subculture.

2.3. Extraction and chromatography of 7-dehydrocholesterol and vitamin D_3 metabolites

A scheme of the procedure used in the extraction, purification and detection of 7-dehydrocholesterol and vitamin D_3 metabolites is outlined in Fig. 1. First, vitamin D_3-related compounds present in *N. glauca* cultured calli (first subculture) and leaves were extracted following essentially the method of Bligh and Dyer [19]. Briefly, the leaves and callus cultures were homogenized with chloro-

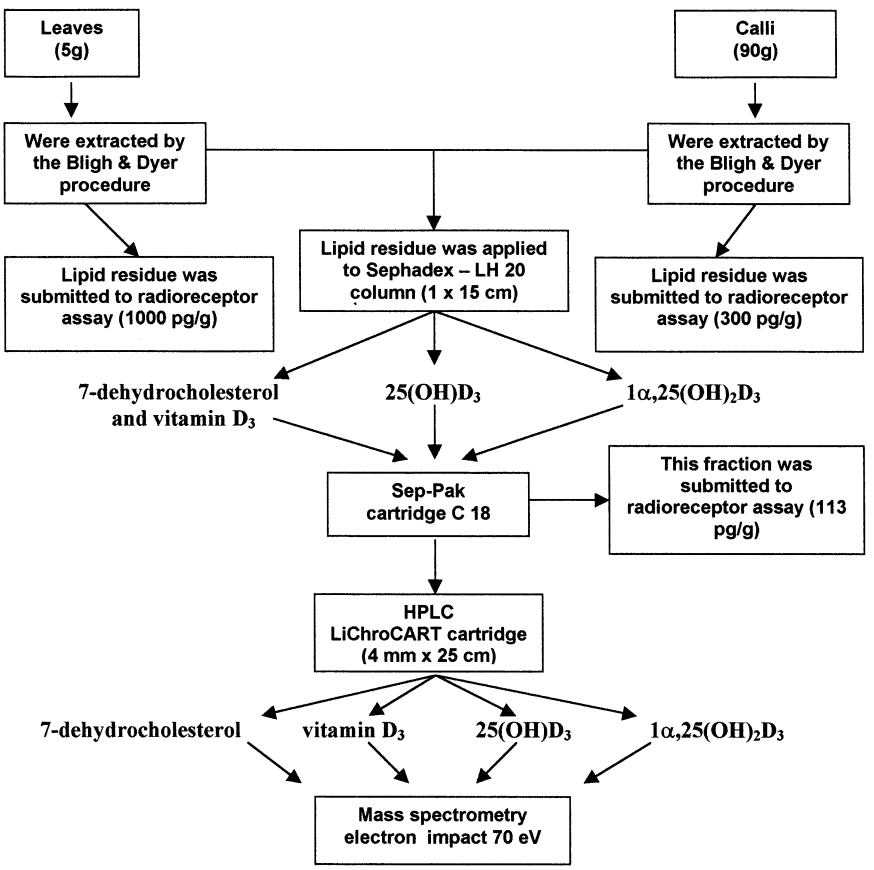

Fig. 1. Flow diagram of the purification and detection of vitamin D_3 metabolites from *N. glauca* leaves and callus cultures. Weight units for leaves and calli refer to g fresh weight (FW). Results of radioreceptor assays are expressed in pg $1\alpha,25(OH)_2D_3$/g FW.

form-methanol (1:2, v/v; 3.6 ml/g FW) using an Ultraturrax homogenizer (Janke and Kunkel, Germany) for 2 min at maximum speed. The samples were further homogenized twice for 30 s: (1) after the addition of chloroform (1.2 ml/g) and then (2) after the addition of water (1.2 ml/g), homogenizing between additions. The final homogenate was centrifuged in a Sorvall SS-34 rotor (Du Pont, Delaware, USA) at $4385 \times g$ for 10 min and the lower lipid soluble phase was collected. When the lipid extract was used for $1\alpha,25(OH)_2D_3$ radioreceptor assays, it was evaporated by flushing nitrogen at 35°C and then dissolved in an appropriate volume of isopropanol. Prior to HPLC, the extracts were passed through a Sephadex LH-20 column (1×15 cm) using hexane-chloroform-methanol (9:1:1, v/v) as the solvent system. The fractions which eluted with similar retention times to the authentic 7-dehydrocholesterol and vitamin D_3 (collected together), $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ were saved. Each fraction from the Sephadex column was passed through a Sep-Pak C 18 cartridge eluted with 90% methanol for 7-dehydrocholesterol and vitamin D_3; 80–100% methanol for $25(OH)D_3$ and 60–80% methanol for $1\alpha,25(OH)_2D_3$, as solvent systems. Fractions were collected which eluted with similar retention times to the authentic 7-dehydrocholesterol and vitamin D_3 (one fraction), $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$. Each fraction was further resolved by high performance liquid chromatography (HPLC). HPLC was performed with an Isco Model 2360 gradient programmer equipped with a Model Isco V^4 multiwavelength programmable detector set at 265 nm and a pump Isco Model 2350 (Isco, Lincoln, NE, USA). A reverse phase LiChroCART (4 mm × 25 cm) cartridge eluted with different solvent systems was used. Vitamin D_3 eluted with methanol-water (86:14, v/v); for eluting 7-dehydrocholesterol, after the vitamin D_3 peak, the solvent was changed to methanol-water (90:10). Isocratic elutions of methanol-water (75:25) and (86:14) were used to isolate $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$, respectively.

2.4. Mass spectrometry

Mass spectra were obtained on a Micromass Model Autospec magnetic sector mass spectrometer, electron impact 70 eV (Micromass, UK). To that end, each HPLC fraction was evaporated and redissolved in isopropanol, except that containing 7-dehydrocholesterol which was solubilized in ethanol. The mass spectra of samples were compared with those of authentic compounds from a database (Wiley 138.1).

2.5. $1\alpha,25(OH)_2D_3$ radioreceptor assays

The $1\alpha,25(OH)_2D_3$ content of Bligh and Dyer [19] lipid extracts from *N. glauca* leaves and callus and the Sep-Pak chromatographic fraction of $1\alpha,25(OH)_2D_3$ derived therefrom was measured by a specific radioreceptor assay. The $1\alpha,25(OH)_2D_3$ receptor preparation was obtained by homogenization of mucosa from duodena of 4-week-old vitamin D-deficient chicks in TEKDP buffer (10 mM Tris–HCl, pH 7.4; 1.5 mM EDTA; 0.3 M KCl; 2 mM dithiothreitol; 0.3 mM phenylmethylsulfonylfluoride) followed by centrifugation at $100\,000 \times g$ for 60 min. The supernatant was collected and stored at −70°C until use. Binding assays were performed incubating the samples with 5.87×10^{-10} M $[^3H]1\alpha,25(OH)_2D_3$ for 4 h at 0°C. Free radioactive steroid was separated from bound by the hydroxylapatite procedure [20].

3. Results

Radioreceptor assays of *N. glauca* leaf and callus lipid extracts revealed the presence of 1000 and 300 pg/g FW, respectively, of $1\alpha,25(OH)_2D_3$. The metabolite was also detected in the corresponding fraction of the Sep-Pak purification step prior to HPLC (Fig. 1).

The presence of vitamin D_3 and its related metabolites (for chemical structures see Ref. [5]) in *N. glauca* was further investigated by high performance liquid chromatography. HPLC analysis on a LiChroCART cartridge of lipid extracts from calli and leaf, previously fractionated by Sephadex LH-20 and Sep-Pak chromatography, revealed the presence of peaks with elution times similar to those of synthetic samples of vitamin D_3 (peak a; 80.1 min), 7-dehydrocholesterol (peak b; 129.0 min), $25(OH)D_3$ (peak c; 34.7 min) and $1\alpha,25(OH)_2D_3$ (peak d; 8.9 min) (Fig. 2, A–C). The above purification scheme has proved to be satisfactory for purification of vitamin D_3 and its derivatives in plant [5,15] as well as in animal

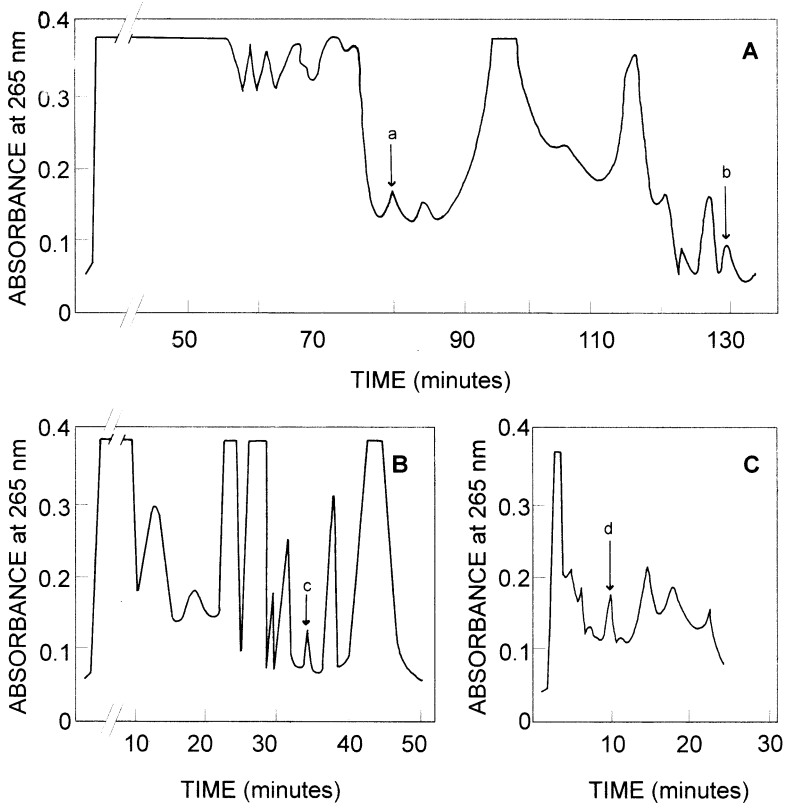

Fig. 2. Isolation of 7-dehydrocholesterol, vitamin D_3 and hydroxylated derivatives from *N. glauca* by HPLC. Lipids were extracted from calli according to Bligh and Dyer [13] followed by chromatography on a Sephadex LH-20 column and a Sep-Pak C 18 cartridge to obtain fractions containing 7-dehydrocholesterol and vitamin D_3, $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ as indicated in Section 2. Each fraction was further resolved by HPLC using a LiChroCART cartridge. (A) Vitamin D_3 and 7-dehydrocholesterol (eluted with methanol/water, 86:14; after 90 min solvent changed to methanol/water, 90:10). (B) $25(OH)D_3$ was eluted methanol/water (75:25). (C) $1\alpha,25(OH)_2D_3$ was eluted methanol/water (86:14); the arrows indicate the elution of authentic samples of: (a) vitamin D_3; (b) 7-dehydrocholesterol; (c) $25(OH)D_3$; and (d) $1\alpha,25(OH)_2D_3$.

tissues [21], prior to their identification by mass spectrometry.

Mass spectrometry of the putative vitamin D_3, 7-dehydrocholesterol, $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ HPLC peaks was performed to elucidate their chemical structures (Fig. 3, A–D). As shown in Fig. 3A, the electron impact mass spectrum of HPLC peak a showed a molecular ion at m/z 384. Loss of water and methyl group from the parent peak gave m/z 351. The peak at m/z 325 was produced by loss of 59 atomic mass units (loss of water plus an isopropyl group) from the molecular ion. The peak at m/z 271 was caused by loss of the side chain from the parent ion. Loss of water from peak m/z 271 gave m/z 253. The peak at m/z 199 was produced by loss of C_4H_8O from peak at m/z 271. The peak at m/z 136 results from formal cleavage between carbons 7 and 8, whereas the base peak at m/z 118 results from dehydration of the peak at m/z 136. Peaks occurring at m/z 118 and 136 are characteristic of the vitamin D-triene system. These peaks and others of Fig. 3A demonstrated that this compound is vitamin D_3.

The mass spectrum of HPLC peak b (Fig. 3B) has a molecular ion at m/z 384. Peaks at m/z 351, 325, 271, 253 and 199 also indicate that the steroid nucleus has remained unchanged. Collectively, from the above data, this metabolite could be 7-dehydrocholesterol.

Peak c of the HPLC run displayed a parent molecular ion of m/z 400 (Fig. 3C) in agreement with the molecular weight of $25(OH)D_3$. Peak at m/z 382 represents loss of water from the molecular ion. Loss of water and methyl group from the parent peak gave m/z 367. The peaks at m/z 271, 253, 136 and 118 arise in the manner discussed above for Fig. 3A. Altogether, these results indicate that the secoesteroid nucleus of their parent, vitamin D_3, has remained unchanged and that this metabolite is formed as a result of changes occur-

ring only on the side chain. From the above data, this metabolite can be identified as 25(OH)D_3.

As shown in Fig. 3D, the mass spectrum of HPLC peak d with a molecular ion at m/z 416 indicates that this metabolite contains one additional hydroxyl group when compared to 25(OH)D_3, in accordance with the structure of 1α,25(OH)$_2D_3$. The peaks at m/z 398 and 380 are characteristic of the parent peak minus one and two molecules of water, respectively. The peak at m/z 287 is due to side chain cleavage from the main steroid molecule (C-17/C-20 cleavage). Loss of the side chain plus one and two molecules of water of the parent ion gives the peaks at m/z 269 and 251, respectively. The peak at m/z 152 results from cleavage between carbons 7 and 8 and represents the A ring plus the carbon 6 and 7 fragment. Loss of water from peak at m/z 152 gives rise to the base peak at m/z 134. Collectively, these peaks and others exhibited in the mass spectrum of Fig. 3D, demonstrate that this metabolite is 1α,25(OH)$_2D_3$.

4. Discussion

The data of this study demonstrate the existence of 7-dehydrocholesterol, vitamin D_3, 25(OH)D_3 and 1α,25(OH)$_2D_3$ in *N. glauca*, a member of the Solanaceae hitherto not known to contain these metabolically related steroids. The presence of vitamin D_3 compounds was established in the lipophilic fraction of this plant species. Various reports have previously shown that in the Solanaceae, the concentration of the free form of vitamin D_3 metabolites generally exceeds that of the corresponding glycosidic derivatives [12,13,22–24]. Moreover, the possibility has been raised that formation of glycoconjugates of vitamin D_3 compounds in plants occurs to a great extent during storage of collected tissues at the expense of the content of free sterols [12,13]. In our study, *N. glauca* leaves or calli were immediately extracted after collection in the field or removal from the culture medium, respectively.

Regarding the identification of vitamin D_3 metabolites, the high specificity of the radiorecep-

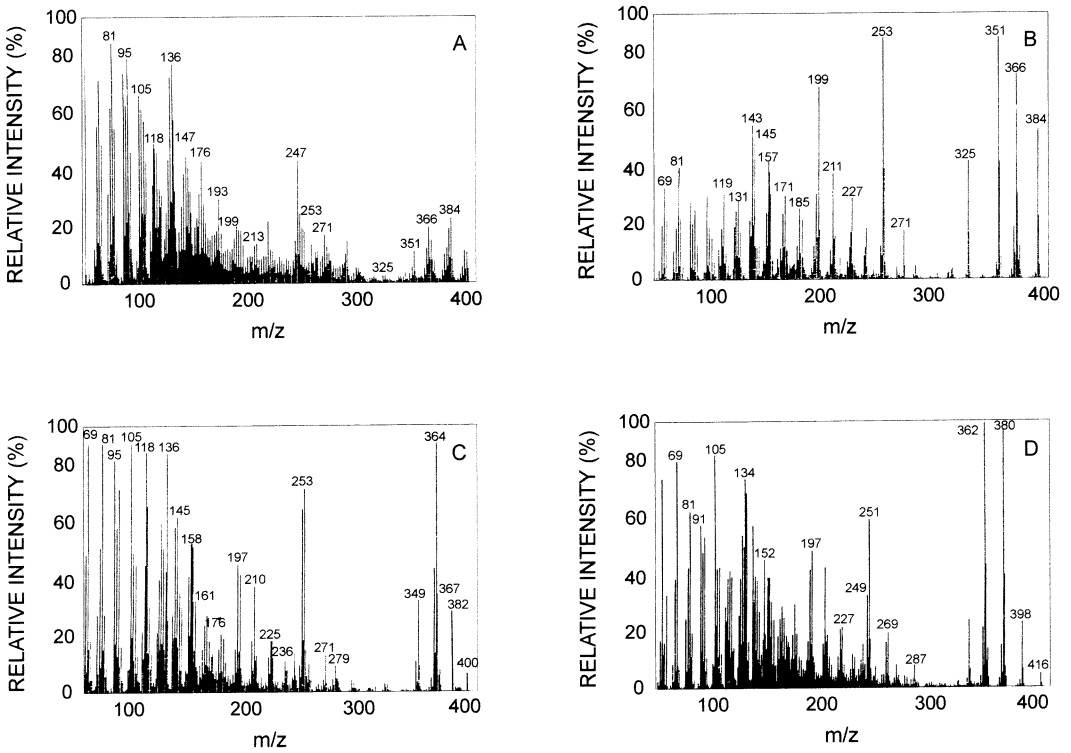

Fig. 3. Mass spectra of HPLC fractions from *N. glauca* containing vitamin D_3, 7-dehydrocholesterol, 25(OH)D_3 and 1α,25(OH)$_2D_3$. The fractions of vitamin D_3-related compounds were obtained by chromatography of *N. glauca* lipid extracts on a Sephadex LH-20 column and a Sep-Pak C 18 cartridge followed by HPLC as in Fig. 1. Mass spectrometry was performed as described in Section 2. (A) Vitamin D_3; (B) 7-dehydrocholesterol; (C) 25(OH)D_3; and (D) 1α,25(OH)$_2D_3$.

tor assay, as revealed by its inability to detect closely related analogs such as $24,25(OH)_2D_3$, $25(OH)D_3$ and $1\alpha(OH)D_3$, even when present in large concentration [25], is clear evidence of the presence of $1\alpha,25(OH)_2D_3$ in leaf and callus cultures from *N. glauca*. More conclusive evidence was furnished by mass spectrometry of the HPLC peak (d) coeluting with $1\alpha,25(OH)_2D_3$, which yielded a molecular ion and a fragmentation profile corresponding to the structure of this metabolite. Also, the presence of 7-dehydrocholesterol, vitamin D_3 and $25(OH)D_3$ in leaf and callus cultures of this plant was confirmed by chromatographic and spectral means, as discussed in Section 3.

This report adds one more example to the growing list of plants shown to contain vitamin D_3 derivatives. Including *N. glauca*, there are so far eight species belonging to the Solanaceae for which biological and/or chemical evidence of the presence of vitamin D_3 and related sterols has been provided. These compounds are also found in varying concentrations in species belonging to other taxonomical families, such as Gramineae, Leguminoseae and Cucurbitaceae [4,13,26].

There is little information about the formation of and the subsequent hydroxylations of vitamin D_3 in plants. Only low activities of vitamin D_3-25-hydroxylase and $25(OH)D_3$-1α-hydroxylase have been detected in *Solanum glaucophyllum* leaf extracts and microsomal membranes using assays analogous to those employed in studies on vitamin D_3 hydroxylation in animals [27]. Much remains to be done to elucidate the metabolism of vitamin D_3 in plants and its regulation.

The fact that the levels of $1\alpha,25(OH)_2D_3$ in leaves and cultured tissue from *N. glauca* are similar to those found in *S. glaucophyllum* [15], a well-known accumulator of vitamin D_3 metabolites, but geographically restricted to certain areas within Argentina and Brazil, indicates that the former species, which is widespread, may represent a more accessible source for investigations on this subject. Finally, the finding that calli from *N. glauca* produce the hormonally active vitamin D_3 metabolite may lead to the use of plant culture systems to produce this compound.

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References

[1] N.A. Worker, B.J. Carrillo, 'Enteque seco', calcification and wasting in grazing animals in the Argentine, Nature 215 (1967) 72–74.
[2] K.M.L. Morris, Plant induced calcinosis: a review, Vet. Hum. Toxicol. 24 (1982) 34–48.
[3] R.H. Wasserman, J.D. Henion, M.R. Haussler, T.A. McCaine, Calcinogenic factor in *Solanum malacoxylon*: evidence that it is 1,25-dihydroxyvitamin D_3-glycoside, Science 194 (1976) 853–854.
[4] R.L. Boland, Plants as a source of vitamin D_3 metabolites, Nutr. Rev. 44 (1986) 1–8.
[5] M.I. Skliar, R.L. Boland, A. Mouriño, G. Tojo, Isolation and identification of vitamin D_3, 25-hydroxy-vitamin D_3, 1,25-dihydroxy-vitamin D_3 and 1,24,25-trihydroxy-vitamin D_3 in *Solanum glaucophyllum* incubated with ruminal fluid, J. Steroid Biochem. Mol. Biol. 43 (1992) 677–682.
[6] A.J. Buchala, A. Schmid, Vitamin D and its analogues as a new class of plant growth substances affecting rhizogenesis, Nature 280 (1979) 230–231.
[7] B.C. Jarvis, A. Booth, Influence of indole-butyric acid, boron, myo-inositol, vitamin D_2 and seedling age on adventitious root development in cuttings of *Phaseolus aureus*, Physiol. Plant. 53 (1981) 213–218.
[8] M. Talmon, R. Vega, R. Boland, Cytohistological studies on the action of vitamin D_3 and stigmasterol on *Phaseolus vulgaris* roots growing in vitro, Plant Sci. 59 (1989) 183–190.
[9] M. Vega, E. Santamaría, A. Morales, R. Boland, Vitamin D_3 affects growth and Ca^{2+} uptake by *Phaseolus vulgaris* roots cultured in vitro, Physiol. Plant. 65 (1985) 423–426.
[10] M. Vega, R. Boland, Vitamin D_3 induces de novo synthesis of calmodulin in *Phaseolus vulgaris* root segments growing in vitro, Biochim. Biophys. Acta 881 (1986) 364–374.
[11] M. Vega, L. Fernandez, R. Boland, Mediation of sterol-induced calmodulin synthesis in *Phaseolus vulgaris* roots by Ca^{2+} and its possible relationship to plant growth regulators, Physiol. Plant. 75 (1988) 499–505.
[12] T.P. Prema, N. Raghuramulu, Vitamin D_3 and its metabolites in the tomato plant, Phytochemistry 42 (1996) 617–620.
[13] T. Aburjai, S. Al-Khalil, M. Abuirjeie, Vitamin D_3 and its metabolites in tomato, potato, egg plant and zucchini leaves, Phytochemistry 49 (1998) 2497–2499.

[14] T. Aburjai, S. Bernasconi, L. Manzocchi, F. Pelizzoni, Effect of calcium and cell immobilization on the production of cholecalciferol and its derivatives by *Solanum malacoxylon* cell cultures, Phytochemistry 43 (1996) 773–776.

[15] A. Curino, M. Skliar, R. Boland, Identification of 7-dehydrocholesterol, vitamin D_3, 25(OH)-vitamin D_3 and 1,25$(OH)_2$-vitamin D_3 in *Solanum glaucophyllum* cultures grown in absence of light, Biochim. Biophys. Acta 1425 (1998) 485–492.

[16] R. Millán, Las especies del género Nicotiana de la flora argentina, Rev. Fac. Agron. Vet. 6 (1928) 169–216.

[17] J.G. Hawkes, R.N. Lester, A.D. Skelding, The biology and taxonomy of the Solanaceae, Linn. Soc. Symp. Ser. 7 (1979) 1–738.

[18] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant. 15 (1962) 473–497.

[19] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.

[20] W.R. Wecksler, A.W. Norman, An hydroxylapatite batch assay for the quantitation of 1,25-dihydroxyvitamin D_3-receptor complexes, Anal. Biochem. 92 (1979) 314–323.

[21] H. Schmidt-Gayk, F.P. Armbruster, R. Bouillon, Calcium Regulating Hormones, Vitamin D Metabolites, and Cyclic AMP. Assays and Their Clinical Application, Springer, Berlin, 1990, pp. 247–279.

[22] T. Aburjai, S. Bernasconi, L. Manzocchi, F. Pelizzoni, Effect of calcium and cell immobilization on the production of cholecalciferol and its derivatives by *Solanum malacoxylon* cell cultures, Phytochemistry 46 (1997) 1015–1018.

[23] T.P. Prema, N. Raghuramulu, Free vitamin D_3 metabolites in *Cestrum diurnum* leaves, Phytochemistry 37 (1994) 677–681.

[24] M. Weissenberg, A. Levy, R.H. Wasserman, Distribution of calcitriol activity in *Solanum glaucophyllum* plants and cell cultures, Phytochemistry 28 (1989) 795–798.

[25] R. Boland, A. Norman, E. Ritz, W. Hasselbach, Presence of a 1,25-dihydroxy-vitamin D_3 receptor in chick skeletal muscle myoblasts, Biochem. Biophys. Res. Commun. 128 (1985) 305–311.

[26] R.L. Horst, T.A. Reihardt, J.R. Russell, J.L. Napoli, The isolation and identification of vitamin D_2 and vitamin D_3 from *Medicago sativa* (alfalfa plant), Arch. Biochem. Biophys. 231 (1984) 67–71.

[27] M. Esparza, M. Vega, R. Boland, Synthesis and composition of vitamin D_3 metabolites, Biochim. Biophys. Acta 719 (1982) 633–640.