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EFFECT OF CALCIUM AND CELL IMMOBILIZATION ON THE PRODUCTION OF CHOLEOCALCIFEROL AND ITS DERIVATIVES BY *SOLANUM MALACOXYLON* CELL CULTURES

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Key Word Index—*Solanum malacoxylon*; *S. glaucophyllum*; Solanaceae; cell suspension cultures; immobilized cells; biosynthesis; biotransformation; cholecalciferol; 25-hydroxycholecalciferol; calcitriol.

Abstract—Cell suspension cultures of *Solanum malacoxylon* cultivated in standard B5 medium containing 1 mM $CaCl_2$ accumulated larger amounts of both vitamin D_3 (cholecalciferol) and 25-OH D_3 (25-hydroxycholecalciferol) than cells in medium containing 10 mM $CaCl_2$. The amount of vitamin D_3 in calcium-free medium was similar to that in standard medium, while the 25-OH D_3 level was 50 time greater; 1α,25$(OH)_2$ D_3 (calcitriol) was detected only in chloroform extracts of cells cultivated in standard medium. It was found that immobilized cells accumulated vitamin D_3 and 25-OH D_3 for prolonged culture periods (up to 30 days) and were able to biotransform vitamin D_3 when it was supplemented as an exogenous precursor. *S. malacoxylon* suspension cell cultures were able to synthesize vitamin D_3, 25-OH D_3 and 1α,25$(OH)_2D_3$ under different experimental conditions, therefore suggesting the presence of enzyme systems able to hydroxylate vitamin D_3 at positions C-25 and C-1. In *Solanum malacoxylon*, as in animals, the concentration of calcium may have an effect on the regulation of vitamin D_3 synthesis and the production of its hydroxylated metabolites. © 1997 Elsevier Science Ltd

INTRODUCTION

Plant cell cultures offer a vital tool for metabolite biosynthesis and biotransformation studies and many attempts have been made to improve yields of desired metabolites and to convert biologically inactive or weakly active compounds into more useful ones [1]. It has been reported that the ingestion of certain plant species causes calcinosis in grazing animals, due to vitamin D-like activity of the leaves [2]. Studies on *Solanum malacoxylon* Sendt. and *Cestrum diurnum* have revealed the active principle to be a glycoside of 1α,25$(OH)_2$ D_3, the hormonally active form of vitamin D_3 in higher animals [3]. The leaves of *S. malacoxylon* have also been reported to contain the glycosides of vitamin D_3 and 25-OH D_3 [4,5].

The production of steroidal alkaloids by callus cultures of several Solanaceae species is well known [6–8], but there have been relatively few reports of induction from wild *Solanum* species of cell suspension cultures able to biosynthetize steroidal metabolites [9]. In a previous paper [10], we reported on the development of callus and suspension cultures from sterile leaves of *S. malacoxylon*, and the detection of cholesterol, 7-dehydrocholesterol, vitamin D_3, 25-OHD_3 and 1α,25$(OH)_2D_3$. We have now studied the effects both of different calcium concentrations in the culture medium and of calcium alginate cell immobilization, on the accumulation of such compounds. The ability of calcium alginate-immobilized cells to biotransform vitamin D_3 added as an exogenous precursor was also investigated.

RESULT AND DISCUSSION

Effect of Ca^{++} concentration

To study the effect of calcium on the level of metabolites related to vitamin D_3 in cell cultures of *S. malacoxylon*, cell suspensions obtained from leaf-derived callus cultures [10] were cultivated in standard liquid B5 medium containing 1 mM $CaCl_2$ (B5.1), in B5 medium containing 10 mM $CaCl_2$ (B5.10) or in Ca^{++}-

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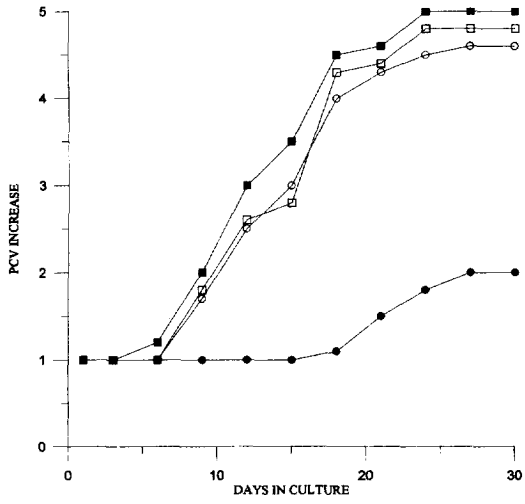

Fig. 1. Growth curves of cell suspension cultures in B5.0 (○), B5.1 (■) and (□) and of alginate-immobilized cells (●).

Table 1. Vitamin D_3, 25-OH D_3 and 1α,25(OH)$_2$ D_3 (μg g^{-1} cell fr. wt) in chloroform extracts from cells (C) and culture media (M). Cultures were grown in B5 media with different Ca^{++} content, as indicated. The analyses were performed 48 hr after inoculation of the cultures

	B5.0		B5.1		B5.10	
	C	M	C	M	C	M
Vitamin D_3	17.3	22.2	25.6	16.5	2.3	5.2
25-OH D_3	26.4	23.3	0.7	0.3	*	*
1α,25(OH)$_2$ D_3	*	*	0.1	*	*	*

* Not detectable.

free medium (B5.0). Cell suspensions were cultivated in all three media in the light on a rotary shaker for 12–96 hours. The modification of the Ca^{++} content in the culture medium had no significant effect on the viability and growth of the cells during this period (Fig. 1). The cells were separated from the culture medium, and both cells and medium were extracted as described in the experimental section. Tests for the recovery of vitamin D_3 that had been added to the three media in the absence of plant cells showed that the different Ca^{++} concentrations in the media did not affect the efficiency of vitamin D_3 extraction. The chloroform extracts (containing free metabolites) were analysed by TLC and HPLC. The identity of vitamin D_3 and 25-OH D_3 was confirmed by mass spectroscopy and chromatographic comparison with authentic samples. Table 1 shows the results of the cell and culture medium analysis 48 hr after inoculum. These data demonstrate that the metabolites were distributed between cells (60–70%) and culture medium.

Cultures grown in B5.10 contained a lower level of vitamin D_3 relative to cultures grown in media of lower calcium content, and no hydroxylated metabolites were detected. Cell cultures cultivated in B5.0 or B5.1 under the same conditions contained not only vitamin D_3, but also 25-OH D_3. 1α,25(OH)$_2$$D_3$ was detected only in B5.1 cultures. In the B5.10 and B5.0 cultures the level of this last metabolite was below the HPLC detection limit, although it could be detected with a radioreceptor assay (unpublished data). These data establish that calcium in the culture medium has an important effect on the accumulation of vitamin D_3 and its metabolites.

The butanol extracts of all the samples were also analysed, after hydrolysis with mixed glycosidases, by TLC and HPLC to investigate the presence of glycosidic forms of the metabolites. Butanol extracts contained D_3 and 25-OH D_3 in much lower amounts but in the same proportions, as the corresponding chloroform extracts. In our cell system, the concentration of free vitamin D_3 metabolites appears to prevail over the corresponding glycosides; similar high concentrations of the aglycone, prevailing over the glycoside forms, have been observed in *Cestrum diurnum* and recently in tomato [11, 12].

Ca-alginate immobilization

Cell immobilization can sometimes be an effective means of increasing secondary metabolite production [13]. We have examined the effect of cell immobilization in calcium alginate [14, 15] on vitamin D_3 and its metabolites. Cell clusters included in alginate beads were cultivated in liquid B5.1 as described. Cell growth was evaluated by determination of packed cell volume (PCV) after dissolving the alginate gel beads in sodium citrate. As shown in Fig. 1, cell growth was reduced in comparison to free suspended cells, but cell viability, tested by fluorescein diacetate (FDA) staining [16] was maintained at good levels (over 70%) for up to 30 days in culture.

Table 2 shows the levels of vitamin D_3 and its hydroxylated metabolites in cultures of calcium alginate-immobilized cells. The metabolites were detected both intracellularly and in the culture medium, in the same proportions as for free suspended cells.

In the absence of specific precursors, the level of vitamin D_3 and its metabolites in cell suspensions in liquid B5 medium, reaches its maximum after 48 hr in culture and then decreases very rapidly [10]. In immobilized cells, however, vitamin D_3 and 25-OH D_3 accumulate for a much longer period and in higher amounts, relative to free suspended cells. The start of

Table 2. Vitamin D_3, 25-OH D_3 and 1α,25(OH)$_2$ D_3 (μg g^{-1} cell fr. wt) in chloroform extracts from alginate immobilized beads (cells and culture medium) at different times (d = days) after inoculation. The culture medium was replaced at day 20

	3 d	6 d	9 d	12 d	15 d	30 d
Vitamin D_3	1.4	73.2	176.8	269.9	9.2	8.4
25-OH D_3	0.6	1.7	4.1	1.2	0.8	7.0
1α,25(OH)$_2$ D_3	*	*	*	0.01	*	*

* Not detectable.

accumulation is strongly delayed in immobilized cells, probably due to a longer lag phase [17]. The rate of production was found to decrease significantly only after 12 days, although cell viability was still quite high. However, when the culture medium was replaced at day 20, a higher relative level of 25-OH D_3 was detected in the medium at day 30. These enhanced levels of vitamin D_3 and 25-OH D_3, compared with free suspended cells in B5.1 medium (Table 1), are in accordance with the already reported stimulation of secondary metabolite production by alginate immobilization of cells [15, 17]. Although the levels of 25-OH D_3 were significantly stimulated by the immobilized cell technique, the recovery of $1\alpha,25(OH)_2D_3$ did not increase. It is noteworthy that, with this technique, plant cells are embedded within an alginate bead matrix, gelled with calcium as divalent cation, but the Ca^{++} in the alginate bead system had no detrimental effect on vitamin D_3 metabolism, probably because the calcium was immobilized.

Our results have demonstrated that the synthesis of vitamin D_3 metabolites takes place in *S. malacoxylon* cell cultures under different experimental conditions. This led us to examine the ability of established cell lines to hydroxylate vitamin D_3, added as an exogenous precursor, in positions 25 and 1α.

Calcium alginate-immobilized cells were employed for the biotransformation experiments. The substrate (8 mg vit. D_3/g cells) was dissolved in a minimal volume of EtOH and enclosed within the calcium alginate matrix together with the cells. The immobilized cells were cultivated in the light on a rotary shaker in B5.1 medium for the indicated periods. Preliminary experiments had indicated that vitamin D_3 did not affect either viability or growth of the immobilized cells. The results of the analysis of cells and culture media by TLC and HPLC are reported in Table 3. The identity of 25-OH D_3, separated and purified as described in the experimental section, was confirmed by ^1H NMR and mass spectroscopy, while $1\alpha,25(OH)_2D_3$ was identified by HPLC analysis in comparison with an authentic sample.

The reported data (Table 3) demonstrate the efficiency of the *S. malacoxylon* immobilized cell system in long term biotransformation experiments. The maximum levels of 25-OH D_3 obtained were over 60 times greater than those detected in the absence of the added precursor (Table 2). The amounts of $1\alpha,25(OH)_2$ D_3 were also increased, although at much lower levels. Our results suggest the presence of an active enzyme able to hydroxylate vitamin D_3 in the 25 position; 1α hydroxylation is also present, although at much lower levels.

Table 3. Biotransformation of Vitamin D_3: 25-OH D_3 and $1\alpha,25(OH)_2$ D_3 ($\mu g\ g^{-1}$ cell fr. wt) in chloroform extracts from alginate immobilized beads (cells and culture medium) at different times (d = days) from the start of the experiment. The culture medium was replaced at day 20

	3 d	6 d	9 d	12 d	15 d	30 d
25-OH D_3	8.3	52.6	254.0	23.0	1.0	4.4
$1\alpha,25(OH)_2$ D_3	*	*	0.1	0.03	*	*

* Not detectable.

EXPERIMENTAL

S. malacoxylon cell cultures. Leaf-derived callus cultures on agar medium were obtained and cultivated in MS-2D medium as previously described [10]. Cell suspension cultures were obtained by transferring calli in the mid-exponential phase (10–15 days from subculture) to liquid Gamborg's B5 medium [18] (B5.1) and cultivated on a rotary shaker (120 rpm) at 25° with a 16 hr photoperiod (light source: Osram L 40 W/20 Sa with a fluence rate of 34 W m^{-2}). Cell growth was estimated by measuring the vol. of sedimented cells (packed cell volume: PCV), and cell viability was evaluated by the fluorescein diacetate (FDA) method [16]. To examine the effects of Ca^{++}, 10–12 g aliquots of cells were grown in 500 ml Erlenmeyer flasks containing 100 ml of culture medium. Modified B5 media were supplemented with 10 mM $CaCl_2$ (B5.10) or deprived of $CaCl_2$ (B5.0). Cells were harvested at 12, 24, 48, 72 and 96 hr.

Immobilization technique. Suspension cultures were decanted in 50 ml plastic centrifuge tubes for 5 min, and the sedimented big cell clusters were discarded. The liquid medium (containing small cell clusters) was gently centrifuged (2000 rpm) for 12 min. The sedimented cells were then resuspended in an equal vol. of B5.1 medium and mixed with a double vol. of sterile 4% Na-alginate soln in H_2O (Sigma). This mixt. was added dropwise with a Pasteur pipette to B5.1 medium supplemented with 100 mM $CaCl_2$, with gentle stirring. After 60 min standing in this soln, the beads formed were rinsed ($\times 3$) in sterile H_2O and once in B5.1 culture medium. In the biotransformation experiments, vitamin D_3 (8 mg in 20 μl EtOH for 1 g cells) was added to the Na-alginate soln, so as to be entrapped with the cells in gel beads. Very low amounts were recovered in bead washings.

Ca-alginate gel beads, containing approximately 5–6 g of cells (fr. wt) were transferred to 500 ml Erlenmeyer flask containing 100 ml B5.1 culture medium. Immobilized cells were cultivated for the indicated periods on a rotary shaker (120 rpm) in the light as described above. At different times, aliquots of alginate beads were dissolved in a 100 mM Na-citrate soln (for 20 min) and PCV and cell viability were evaluated by fluorescence microscopy after FDA staining. The culture medium was replaced with fresh medium after 20 days.

Extraction and analysis. Free suspended cells or alginate-immobilized cells were harvested at the indicated periods. Cells and culture medium were sepd by centrifugation (2400 rpm for 20 min) or by filtration (for immobilized cells), and extracted separately. Cells were ground in the presence of liquid N_2 and macer-

ated for 10 min with aq. 10% NH_4OH (10 ml), then extracted $\times 3$ with 30 ml $CHCl_3$–MeOH (2:1) and $\times 3$ with 30 ml *n*-BuOH. The culture medium was extracted $\times 3$ with 30 ml $CHCl_3$ then $\times 3$ with 30 ml *n*-BuOH. The solvents were removed under red. pres. The BuOH extracts were hydrolysed enzymically using a mixed glycosidases prep. derived from *Turbo cornutus* (ICN Biomedicals, Inc.) in a pH 4 buffer at 37° for 72 hr and then extracted ($\times 3$) with 15 ml $CHCl_3$. The organic phase was dried with Na_2SO_4, filtered and the solvent removed under red. pres. $CHCl_3$ extracts were analysed by TLC/HPLC and quantified by HPLC (Nova-Pak C18, 150×3.9 mm). A gradient elution was performed [MeOH–H_2O (3:1) for 15 min and then 15 min to 100% MeOH] at a flow rate of 1 ml min^{-1}. Vitamin D_3, 25-OH D_3 and 1α,25$(OH)_2$ D_3 were identified by UV monitoring at 264 nm and by comparing their R_fs with those of authentic samples. The metabolites were quantitated by comparison of the sample peak areas with the standard.

From the $CHCl_3$ extracts of alginate immobilized cells, vitamin D_3 was purified and identified as described [10]. The $CHCl_3$ extracts of immobilized cells, in the experiment with exogenous vitamin D_3, were partially purified by flash-chromatography (Merck Kiesegel 230–400 mesh) using petrol–EtOAc (7:3) and then (3:7). The frs from the last column corresponding to 25-OH D_3 were collected and further rechromatographed (prep. TLC; $CHCl_3$–MeOH 19:1). The fr. corresponding to 25-OH D_3 was scraped off and extracted ($\times 3$) with $CHCl_3$, $CHCl_3$–MeOH (1:1) and MeOH. The combined organic solvents were evapd under red. pres. The chemical identity of 25-OH D_3 was confirmed by ^1H NMR and mass spectroscopy and by comparison with published data [18, 19].

Instrumentation. ^1H NMR: Ac-200 Bruker (200 MHz); MS: VG 7070 EQ.

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