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Bioproduction of diosgenin in callus cultures of *Trigonella foenum-graecum* L.

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Abstract

The production of the steroidal sapogenin, diosgenin, by callus cultures of *Trigonella foenum-graecum* L. (fenugreek) is described. The levels of this secondary compound were examined by high performance liquid chromatography with a diode-array detector, and its identity was confirmed by mass spectrometry. The levels of diosgenin detected in leaf callus exceeded the levels detected in stem and root calli. The diosgenin levels accumulated in leaf, stem and root calli at 45 days (maximum production) represent 22, 10 and 27%, respectively, of the levels detected in the corresponding organs of the mother plant at 45 days. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Callus culture; Diosgenin; Steroidal sapogenin; *Trigonella foenum-graecum*

1. Introduction

Diosgenin is a steroidal sapogenin belonging to the triterpene group and is of great interest to the pharmaceutical industry because of its oestrogenic effect on the mammary gland (Aradhana, Rao & Kale, 1992). It also plays an important role in the control of cholesterol metabolism (Cayen & Dvornik, 1979; Holland, Rahman, Morris, Coleman & Billington, 1993; Marzolo & Nervi, 1989; Roman, Thewles & Coleman, 1995; Sauvaire, Ribes, Baccou & Loubatieres-Mariani, 1991), produces changes in the lipoxygenase activity of human erythroleukaemia cells (Nappez, Liagre & Beneytout, 1995), and is responsible for morphological and biochemical changes in megakaryocyte cells (Beneytout, Nappez, Leboutet & Malinvaud, 1995). Diosgenin is mainly used as starting material for partial synthesis of oral contraceptives, sex hormones and other steroids (Zenk, 1978).

In a previous study we found that the maximum levels of this secondary compound in *Trigonella foenum-graecum* L. plants are reached in young leaves, lower levels being detected in stems and roots (Ortuño, Oncina, Botia & Del Rio, 1998). Curiously, most of the studies concerning the expression of this sapogenin in the plant material studied here have concentrated on root cultures (Rodríguez-Mendiola, Stafford, Cresswell & Arias-Castro, 1991; Zafar & Garg, 1990), hairy root cultures (Merkli, Christen & Kapetanidis, 1997), and its expression in seeds (Bohannon, Hagemann, Earle & Barclay, 1974). Little mention has been made of diosgenin's expression in plant leaves (Dixit, Laxmi, Srivastava, Gupta, Sircar & Bhatt, 1985; Taylor et al., 1997; Varshney, Jain & Srivastava, 1984).

In vitro production of this secondary compound is an alternative way to obtain this important sapogenin, so that its supply (especially on an industrial scale) is not dependent on environmental conditions or on a particular growth stage of the plant in question. The results obtained in this respect using tissue cultures of *Trigonella foenum-graecum* are contradictory, some authors detecting levels of diosgenin which were higher than those observed in the initial seeds (Khanna & Jain, 1973) and others finding no significant amounts under similar conditions (Brain & Lockwood, 1976). However, such differences may be simply due to not using an analytical method suitable for the identification and quantification of this compound.

The resolving power of high performance liquid chromatography (HPLC), together with mass spectrometry, is used as an identification and quantification procedure in the production of diosgenin in callus cultures

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from *Trigonella foenum-graecum*. The accumulation of this steroidal sapogenin during different stages of growth in leaf, stem and root callus cultures was analysed and compared with the levels found in the corresponding organs of the mother plant.

2. Materials and methods

2.1. Plant material

Trigonella foenum-graecum seeds were supplied by Plantaforma, León (Spain), and sterilised by soaking in 100% ethanol for 1 min and then in a 20% v/v Domestos® solution for 15 min. They were rinsed several times in sterile water before being implanted on a 0.7 % w/v agar medium. Cultures were maintained at 25°C with continuous light. After 2 or 3 days the germinated seeds were transferred to Murashige and Skoog (1962) culture medium containing naphthalene-acetic acid (NAA) (3×10^{-6} M) and 0.7% agar at pH 5.8.

Three to four weeks after germination the different plant organs (leaves, stems and roots) were separated and placed separately in different culture media for the corresponding calli to proliferate.

2.2. Callus culture and measurement of growth

Six different culture media were used to grow the explants. The first (Type I) was composed of the basic Murashige and Skoog (1962) medium supplemented by coconut milk (collected from fresh mature coconut) (150 ml l^{-1}); Type II contained the same basic medium but supplemented by malt extract (Sigma) (500 mg l^{-1}); Type III used White's basal medium (White, 1943) supplemented by coconut milk (150ml l^{-1}); Type IV used White's basal medium supplemented by malt extract (500 mg l^{-1}); Type V used Gamborg's medium (Gamborg & Wetter, 1975) supplemented by coconut milk (150 ml l^{-1}); and Type VI used Gamborg's medium supplemented by malt extract (500 mg l^{-1}). Agar (0.7%) and NAA (3×10^{-6} M) were added in all cases and the pH adjusted to 5.8.

All types of media were sterilized at 121°C for 20 min.The calli were grown and maintained in a culture chamber at 25°C with a 16-h light photoperiod, provided by fluorescent tubes (Sylvania, Gro-Lux, F36W/Gro, 5000 lux). Tissues were subcultured at 3 week intervals.

Three times subcultured calli were used to measure growth and the fresh and dry weight were determined at different times after subculturing.

2.3. Isolation and measurement of diosgenin

At different times, 2 g of the calli were used for the isolation of dioscin, which was then hydrolysed to diosgenin following the method of Sauvaire and Baccou (1978) and optimised for our work conditions (Ortuño et al., 1998). The analyses were performed with a Hewlett Packard liquid chromatograph (model HP 1050) with a diode-array detector (range scanned: 190–500 nm). Reverse phase chromatographic separation was carried out on a μBondapak C_{18} (5 μm, 250×4.6 mm i.d.) column isocratically, using a mixture of acetonitrile: water (90:10 v/v) at 1 ml/min at 35°C. Changes in absorbance at 214 nm were recorded. Diosgenin (R_t = 12.27 min) was quantified by comparison with an external standard. The identity of diosgenin was confirmed by its mass spectrum (Hewlett Packard Mass Spectometer model 5989), as described in our previous paper (Ortuño et al., 1998).

2.4. Chemicals

Diosgenin was purchased from Sigma.

3. Results and discussion

3.1. Callus growth studies

Table 1 shows the relative percentage of growth of the root calli in the first stage (callogenesis) and in the growing stage. As can be seen, optimum growth in both stages was obtained when Murashige and Skoog's (MS) medium was used, Medium Types I and II giving 100 and 77.58% callogenesis, while the other media led to callogenesis of around 50%. The results also show that coconut milk is a better supplement than malt extract since the callus grown in Type I (supplemented with coconut milk) grew approximately 20% more than in Type II (supplemented with malt extract). Subsequent callus growth was also greater in Type I than in the other media used.

Table 1 also shows that Type I was the best medium for both stages of callus growth of the stem. Type V was also a suitable medium for callogenesis (98%), but cannot be recommended for subsequent growth (3%). The same results confirm the superiority of coconut milk as a supplement since the callogenesis achieved with this supplement (Type V, 98%) exceeded that achieved with the same basic medium supplemented by malt extract (Type VI, 56%).

Type I also achieved the best results in leaf callus cultures since, all the other media led to callus growth rates substantially below 50%.

After deciding on the most suitable growth medium for *Trigonella foenum- graecum* callus growth (medium Type I, Table 1), we made an exhaustive study of the growth of the three callus types (root, stem, leaf). For leaf calli, Fig. 1 shows an exponential growth phase from 15 to 28 days, followed by a linear phase up to about 45 days, when the stationary phase started. For

Table 1
Relative percentages of root, stem, and leaf callus growth in first stage (callogenesis) and growth stage in different culture media. Compositions of the different culture media: **I** and **II**: basic medium Murashige and Skoog + coconut milk (**I**) or malt extract (**II**); **III** and **IV**: basic medium White + coconut milk (**III**) or malt extract (**IV**); **V** and **VI**: basic medium Gamborg + coconut milk (**V**) or malt extract (**VI**). Included in all culture media: NAA in the concentration described in Materials and methods

Growth stage	Culture media					
	I	II	III	IV	V	VI
Root						
Callogenesis	100%	77.58%	46.55%	51.72%	48.30 %	51.72%
Callus growth	100%	4.44%	2.18%	1.44%	1.25%	1.33%
Stem						
Callogenesis	100%	65.00%	43.75%	68.75%	97.60%	56.25%
Callus growth	100%	14.61%	2.15%	3.07%	3.03%	1.80%
Leaf						
Callogenesis	100%	73.33%	53.30%	30.00%	24.00%	23.30%
Callus growth	100%	43.83%	7.21%	4.29%	5.57%	3.26%

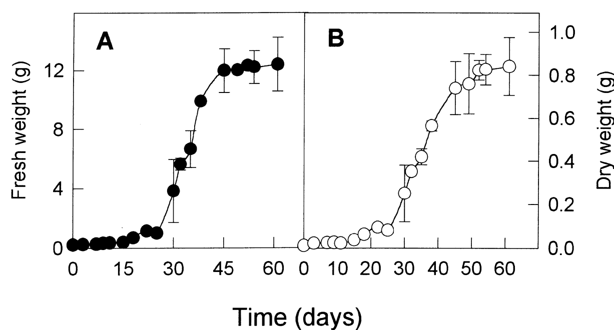

Fig. 1. Growth of *Trigonella foenum–graecum* leaf callus cultures. A, Fresh weight (g) and B, Dry weight (g). Experiments were replicated three times. Vertical bars denote ±S.E.

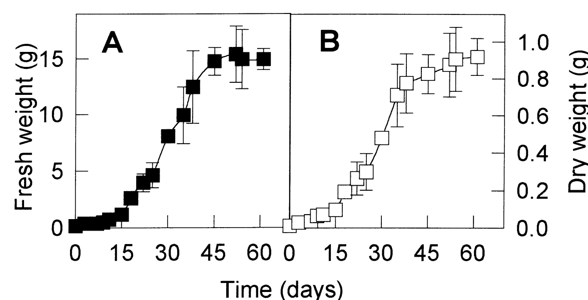

Fig. 2. Growth of *Trigonella foenum–graecum* stem callus cultures. A, Fresh weight (g) and B, Dry weight (g). Experiments were replicated three times. Vertical bars denote ±S.E.

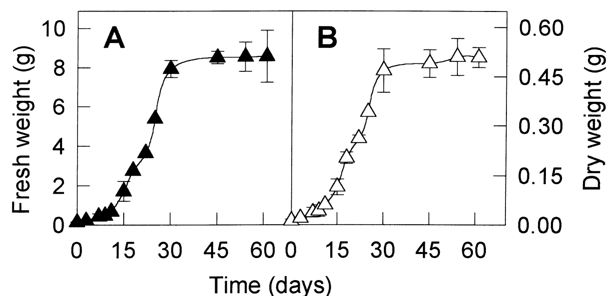

Fig. 3. Growth of *Trigonella foenum–graecum* root callus cultures. A, Fresh weight (g) and B, Dry weight (g). Experiments were replicated three times. Vertical bars denote ±S.E.

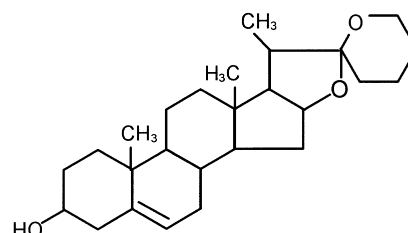

Fig. 4. Chemical structure of diosgenin.

stem calli (Fig. 2), the corresponding values were 12–20 days (exponential phase) and 20–45 days (linear phase), followed by the stationary phase. For root calli (Fig. 3) the exponential phase lasted from 10–15 days and the linear phase from 15 to 30 days, followed by the stationary phase.

3.2. Diosgenin content in callus cultures

The results show that the diosgenin levels (see Fig. 4 for its structure) accumulated at all the ages analysed (15, 27, 37, 45 and 60 days) were higher in leaf calli (1.3, 1.9, 2.0, 2.2 and 1.8 mg, respectively) than in stem calli (0.10, 0.34, 0.60, 0.74 and 0.60 mg, respectively) and root calli (0.06, 0.37, 0.41, 0.60 and 0.55 mg, respectively) (Fig. 5). These findings reflect our previous observations at plant level (Ortuño et al., 1998). Diosgenin levels in leaf calli at the end of the linear phase of growth were about 3–5 times the levels observed in the calli from other plant organs. In all three callus types (Fig. 5), maximum levels were attained after 45 days, which coincided with the onset of the stationary phase of growth for both leaf and stem cultures (see Figs. 1–3).

These results suggest that leaf calli are the most suitable starting material for obtaining diosgenin in vitro. The results also show that all three callus types (leaf, stem and root) of *Trigonella foenum-graecum* obtained in our culture conditions, accumulate diosgenin in the same way as they do in the mother plant (Ortuño et al., 1998), implying that under our conditions there has been no alteration in the metabolic pathway as a result of in vitro cultivation.

The diosgenin levels accumulated in leaf, stem and root calli at 45 days (maximum production) represent 22, 10 and 27% of the levels detected in the corresponding organs of the mother plant at 45 days (Ortuño et al.,

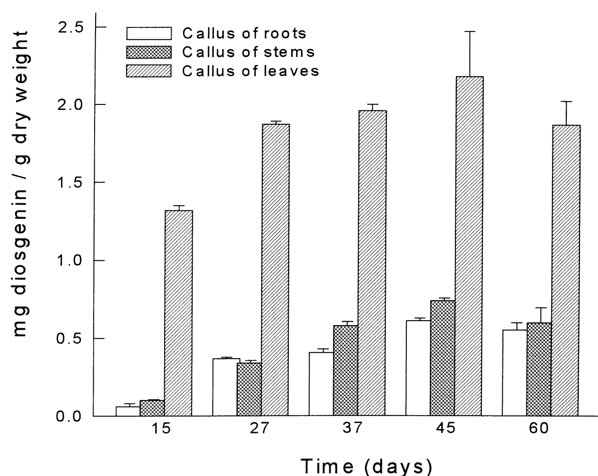

Fig. 5. Time course of diosgenin levels in calli of roots, stems and leaves, obtained from explants of *Trigonella foenum–graecum*. Experiments were replicated three times. Vertical bars denote ±S.E.

1998). These lower levels reflect the results obtained by us and other authors for the bioproduction of several secondary metabolites (Barthe, Jourdan, McIntosh & Mansell, 1987; Del Río & Ortuño, 1994; Del Río, Orturio, Garcia Puig, Iborra & Sabater, 1991; Del Río, Ortuno, Marin Garcia Puig & Sabater, 1992; Parr, 1989). Bearing in mind that, at mother plant level, it is possible to modulate the levels of diosgenin by different plant growth regulators (Ortuño et al., 1998, 1999), we are now carrying out experiments to ascertain whether the same treatments produce parallel effects in the levels of diosgenin accumulated in the calli studied here.

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