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Food Chemistry 76 (2002) 475–479

Food Chemistry

www.elsevier.com/locate/foodchem

Effect of ethylene on diosgenin accumulation in callus cultures of *Trigonella foenum-graecum* L.

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Received 22 May 2001; received in revised form 8 August 2001; accepted 8 August 2001

Abstract

This paper studies the effects of different concentrations of ethephon (an ethylene-releasing compound) on the level of the steroidal sapogenin, diosgenin, in callus cultures of *Trigonella foenum-graecum*. The results show that diosgenin synthesis and/or accumulation was stimulated by 5, 15, and 25 ppm ethephon. This was particularly true in the case of the 25 ppm treatment when an increase of 195% was observed. Concentrations of 50 ppm and 100 ppm reduced diosgenin levels. The following morphological and ultrastructural changes were observed in the callus cells of *T. foenum-graecum* treated with ethephon: increased cell diameter, decreased cell packing (for all concentrations assayed), increased cytoplasmatic density (25 ppm treatment) and alteration of the membrane structures (50 and 100 ppm). © 2002 Published by Elsevier Science Ltd.

Keywords: Callus growth; Ethephon; Diosgenin biosynthesis modulation; Steroidal sapogenin

1. Introduction

Diosgenin, a steroidal sapogenin belonging to the group of triterpenes and an aglycone of dioscin, is a very important compound in the pharmaceutical industry, which uses large quantities of diosgenin-type substances to produce corticoids and contraceptives in chemical and biological processes (Vezina, 1987). Furthermore, the versatility of this steroidal sapogenin means it can be efficiently transformed into progesterone and Reichsteins S compound, not to mention C_{19} intermediaries for the synthesis of androgens, estrogens and various 19-nor-steroid substances (Vezina, 1987).

The seeds of *Trigonella* have been seen to have antidiabetic properties (Al-Habbori & Raman, 1998) and they are widely used in experimental studies and treatments of this disorder (Baquer, Gupta, & Raju, 1998; Genet, Kale, & Baquer, 1999; Gupta, Raju, & Baquer, 1999). They also have hypercholesterolemic properties both for animals and humans (Vezina, 1987). Both properties have been attributed to the presence of steroidal sapogenins, such as diosgenin, observed in extracts of its seeds (Vezina, 1987). This compound plays an important role in the metabolism of cholesterol (Cayen & Dvornik, 1979; Holland, Rahman, Morris, Coleman, & Billington, 1993; Marzolo & Nervi, 1989; Sauvaire, Ribes, Baccou, & Loubatières-Mariani, 1991; Roman, Thewles, & Coleman, 1995), as has been seen in diabetic rats fed with *Trigonella* seeds, in which the LDL and VLDL fractions of total cholesterol decreased and the HDL-cholesterol increased. Another effect, related to the metabolism of cholesterol, is increased biliary secretion of cholesterol after treatment with diosgenin, which has been seen to have a cell-protecting effect in rat liver (Accatino, Pizarro, Solis, & Koening, 1998) thus preventing hepatic damage (Amigo, Mendoza, Zanlungo, Miquel, Rigotti, González, & Nervi, 1999). Transport studies of biliary cholesterol in rats show that biliary salts are normally used up but that diosgenin treatment prevents this because of the biliary cholesterol it produces. Thus, treatment with diosgenin significantly reduces levels of biliary alkaline phosphodiesterase (Thewles, Parslow, & Coleman, 1993).

Diosgenin has also been described as having anti-inflammatory properties (Chiang, Tseng, Wang, Chen, & Kan, 1991; Wu, Lin, Chiang, Horhg, & Chung, 1990) and as having antitumoral activity since it has been shown to be cytotoxic both in vivo and in vitro experiments (Chiang et al., 1991; Wu, Chiang, Fu, Chien, Chun, & Horng, 1990; Wu et al., 1990).

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0308-8146/02/$ - see front matter © 2002 Published by Elsevier Science Ltd.
PII: S0308-8146(01)00309-0

In previous studies using plants of *Trigonella foenum-graecum* for the bioproduction of diosgenin, it was observed that the highest level of this steroidic sapogenin are expressed in leaves (Ortuño, Oncina, Botía, & Del Río, 1998). However, an alternative way of producing it is the in vitro culture of cell systems. Indeed, we have shown that, as occurs in the mother plant, the levels of diosgenin thus-produced in *T. foenum-graecum* leaf calli are 3–5 times greater than in stem or root calli (Oncina, Botía, Del Río, & Ortuño, 2000).

As shown by several authors, growth regulators may be involved in the secondary metabolism of plants, where they inhibit or activate the biosynthesis of different secondary metabolites, such as terpenes (Coggings, Scora, Lewis, & Knapp, 1969; García Puig et al., 1993; Ortuño, García Puig et al., 1993; Ortuño, Oncina, Botía, & Del Río, 1998; Wilson, Shaw, McDonald, Greany, & Yokohama, 1990), phenols (Berhow & Vandercook, 1992; Del Río, Fuster, Sabater, Porras, García Lidón, & Ortuño, 1995; García Puig et al., 1995) and alkaloids (Cho, Kim, & Pedersen, 1998). We have shown that ethylene plays an important role in regulating diosgenin synthesis and/or accumulation in *T. foenum-graecum* plants (Ortuño et al., 1999).

The objective of this study was to see the effect of different concentrations of this growth regulator on the expression of diosgenin in callus cultures of *T. foenum-graecum* and its relationship with possible alterations in cell growth during these treatments.

2. Material and methods

2.1. Plant material, hormonal treatments and measurement of growth

The *T. foenum-graecum* seeds were supplied by Plantafarma, León (Spain). Sterilisation, seedling growth and the establishment and growth of calli followed the procedure described in a previous paper (Oncina, Botía, Del Río, & Ortuño, 2000). Hormonal treatment involved infiltrating different quantities of ethephon (5, 15, 25, 50 and 100 ppm) into the culture media after autoclaving and before the agar gelled. Leaf calli were subcultured in these new culture media, after determining the weight of the callus transferred, as that growth could be calculated from subsequent determinations. At the same time, the dry weights of similar fresh callus fragments were determined. The culture process was carried out in a chamber at 25 °C with a 16-h photoperiod, supplied by fluorescent tubes (Sylvania, Gro-Lux, F36W/Gro, 5000 lux).

Measurements of growth were made after 40–45 days when the stationary stage of growth was reached. The fresh and dry weight of control calli and of those grown in the different concentrations of ethephon were calculated.

2.2. Light and electron microscopy

The microscopic studies made and the conditions and procedures used to process the *T. foenum-graecum* leaf calli are those described in previous studies (Del Río, Ortuño, García Puig, Iborra, & Sabater, 1991; Ortuño, Sánchez-Bravo, Moral, Acosta, & Sabater, 1990), where the ways of obtaining the corresponding semithin and ultrathin sections for optical and electronic microscopy are also detailed. Cell diameter and density were measured by using an ocular micrometer and an integration plate coupled to a Photomicroscope II (Carl Zeiss, Oberkochem, FRG), respectively. For the ultramicroscope studies, the callus sections were cut (Ultracut Reicher Jung) and stained with uranyl acetate and lead citrate before being examined in a Zeiss EM 109 microscope with an acceleration of 60 kv. The quantification of the number of free cytoplasmatic ribosomes in the control and 25 ppm ethephon treated calli was carried out by the morphometric analysis, according to the procedure described in a previous paper (Ortuño et al., 1990).

2.3. Isolation and measurement of diosgenin

For the isolation of dioscin and its subsequent hydrolysis to diosgenin, 2 g of the calli were used, following the procedure described in a previous paper (Ortuño et al., 1998). The analyses were performed with a Hewlett Packard liquid chromatograph (model HP

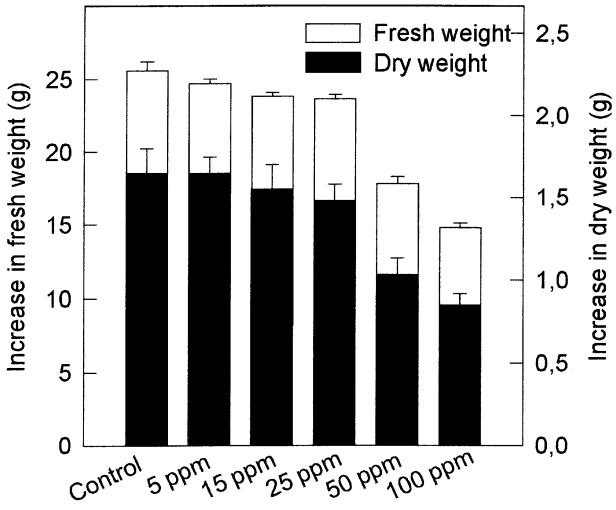

Fig. 1. Effect of ethrel on the growth of *Trigonella foenum-graecum* leaf calli. After 45 days the increase in fresh and dry weight (g) of the calli cultivated with different concentrations of ethrel were compared with the control. The measurements were made in triplicate and the bars represent ± S.E. ($n = 3$).

1050) with a diode-array detector (range scanned: 190–500 nm). Reverse phase chromatographic separation was carried out on a μBondapak C_{18} (250×4.6 mm i.d.) column, isocratically, using a mixture of acetonitrile:-water (90:10; v/v) at a flow of 1 ml/min at 35 °C. Changes in absorbance at 214 nm were recorded. Diosgenin was quantified by comparison with an external standard. The identity of diosgenin was confirmed by reference to 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 (USA); ethephon [commercial Ethrel, 48% (2-chloroethyl) phosphonic acid] was from Etisa (Spain).

3. Results and discussion

3.1. Influence of ethephon treatments on callus growth

The addition of different concentrations of ethrel to the culture medium produced a slight delay in the growth of the *T. foenum-graecum* leaf callus cultures (Fig. 1), which was commensurate with the concentration of ethrel used. With the 5, 15 and 25 ppm treatments, the reduction was about 3% (5 ppm) or 6% (15 and 25 ppm), while 50 and 100 ppm led to reductions in weight of 25 and 35%, respectively (Fig. 1).

Optical microscopic study showed that the size of the callus cells grown in supplemented medium increased, while the cell packing (number of cells/10^4 μm^2) had decreased (Table 1).

As can be seen from the same table, the increase in size reflected the ethrel concentration used (20% increase with 25 ppm and 50% with 50 and 100 ppm). The reduction in cell packing was about 20% with 25 ppm and 35% with 50 and 100 ppm).

3.2. Effect of ethephon treatments on the biosynthesis of diosgenin in callus cultures

The levels of diosgenin were higher in the callus cultures grown with ethrel in the culture medium than in the controls, the 5, 15 and 25 ppm treatments leading to increases of 20.4, 38.2 and 195.4%, respectively (Fig. 2). However, the further increases in ethrel concentration led to falls in diosgenin biosynthesis of 70 and 80.5% for 50 and 100 ppm, respectively.

Taking all the above into consideration, the best results as regards cell growth (Fig. 1) and diosgenin biosynthesis/accumulation (Fig. 2) were obtained with 25 ppm ethrel (170% increase in diosgenin compared with control).

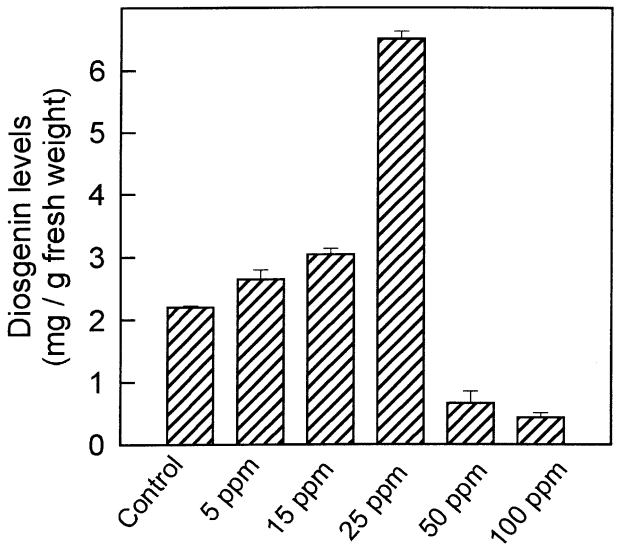

Fig. 2. Diosgenin levels in leaf calli of *Trigonella foenum-graecum* grown in control medium and media with different concentrations of ethrel. The measurements were made in triplicate and the bars represent ± S.E. ($n = 3$).

Table 1
Effect of ethrel on cell size and packing in *Trigonella foenum-graecum* leaf calli after 45 days in control and ethrel-supplemented media[a]

	Cell size (μm)		Packing (number of cells/10^4 μm^2)
	Longitudinal	Equatorial	
Control	41.8±7.3	39.2±5.5	7.17±1.74
Ethrel 25 ppm	50.0±7.6	49.3±0.6	5.77±1.36
Ethrel 50 ppm	64.0±4.6	51.3±2.5	4.65±1.07
Ethrel 100 ppm	65.7±13	57.7 ±10	4.8±0.6

[a] The data correspond to cell diameter (longitudinal and equatorial) in μm. Cell packing is expressed as number of cells in 10^4 μm^2. The data correspond to mean values ± S.E. ($n = 3$).

An ultrastructural study of the *Trigonella* leaf callus cultures (both control and treated) showed that high ethrel doses (50 and 100 ppm) breaks and/or disorganises the membranes and membrane systems (Fig. 3D) and leads to the structural disorganisation of the organelles (Fig. 3D); this accords with the lower growth of the calli treated with ethrel (Fig. 1) and would also explain the lower levels of diosgenin seen in calli grown in these media (Fig. 2), since the final enzymes of the biosynthetic pathway of this sapogenin (oxidosqualene cyclase and those responsible for transforming cycloartenol into cholesterol and this into diosgenin) are linked to the membranes. In the case of calli, grown in 25 ppm ethrel, the cells were seen to be very vacuolised (Fig. 3C), much like the meristem cells with a high metabolic activity. They also showed a greater cytoplasmatic density (Fig. 3B) than the controls (Fig. 3A). This high density may be due to the high number of free cytoplasmatic ribosomes, which are incremented by 15% compared to

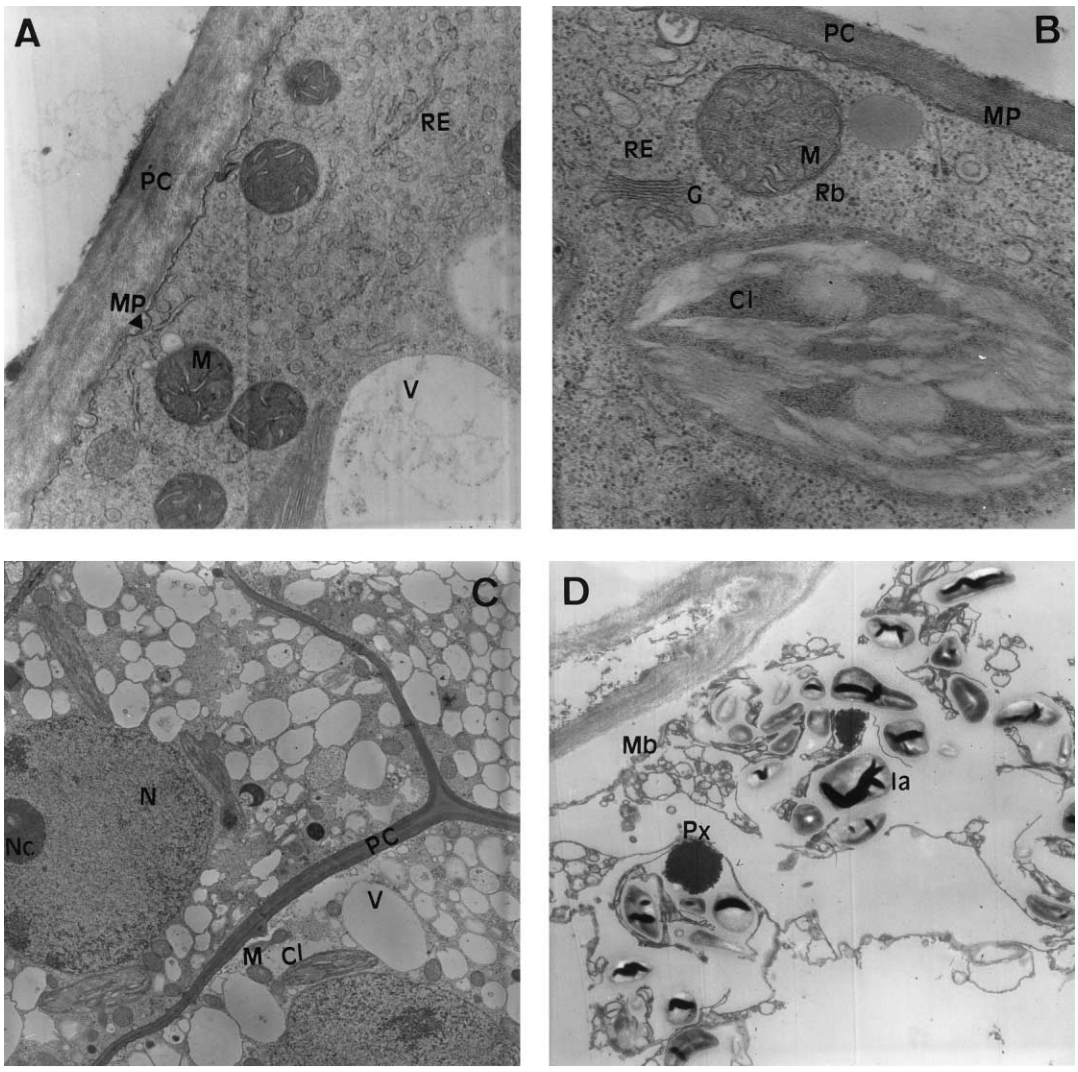

Fig. 3. Electronic micrographs of cells in leaf callus cultures after 40 days. A, control callus cells ×18 400; B y C, cells of calli grown in media with 25 ppm ethrel (×32 000 and ×4590, respectively); D, cells of calli grown in media with 100 ppm ethrel, ×6400). CW, cell wall. M, mitochondria. Px, peroxisome. Rb, ribosomes. PM, plasmatic membrane. AI, amyliferous inclusions; Mb, membrane; ER, endoplasmic reticulum; G, golgi body; V, vacuole; Cl, chloroplast; N, nucleus; Nc, nucleolus.

the control. These are involved in enzyme synthesis in the peroxisome, an organelle which is very important for the synthesis of terpenic compounds, since it contains the enzymes involved in the first stages of the biosynthesis of these compounds (Biardi & Krisans, 1996), such as mevalonate-5-phosphotransferases on mevalonate kinase (Biardi, Sreedhar, Zokaei, Vartak, Bozeat, Shackelford, Keller, & Krisans, 1994; Stamellos, Shackelford, Tanaka, & Krisans, 1992), phosphomevalonate kinase, or mevalonate-5-phosphate kinase (Chambliss, Slaughter, Schriner, Hoffman, & Gibson, 1996), mevalonate-5-pyrophosphate decarboxylase and farnesyl pyrophosphate synthase (Stamellos, Shackelford, Tanaka, & Krisans, 1992). These results would explain the increase observed in the diosgenin levels of cells treated with 25 ppm ethrel (Fig. 2).

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