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Distribution and changes of diosgenin during development of *Trigonella foenum-graecum* plants. Modulation by benzylaminopurine

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The distribution and evolution of diosgenin levels in the different organs of *Trigonella foenum-graecum* were investigated. The results revealed that the maximum levels of this secondary compound are reached in the young leaves (20 mg g^{-1} dry weight). A considerable increase in the levels of this sapogenin was observed after treatment of seeds with benzylaminopurine. These results open up new perspectives for the possible development of plants with high concentrations of this compound. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Dioscin and its aglycone, diosgenin, are of great interest pharmaceutically, since the former can be used as an anti-inflammatory or anti-neoplastic agent (Wu *et al.*, 1990), shows antiviral activity (Aquino *et al.*, 1991) and cytotoxicity towards some cancer cell lines and has immunomodulating effects (Chiang *et al.*, 1991). Diosgenin has an estrogenic action on the mammary gland (Aradhana *et al.*, 1992), plays an important role in the control of cholesterol metabolism (Cayen and Dvornik, 1979; Marzolo and Nervi, 1989; Sauvaire *et al.*, 1991; Holland *et al.*, 1993; Roman *et al.*, 1995), and produces changes in lipoxygenase activities in human erythroleukemia cells (Nappez *et al.*, 1995) and morphological and biochemical changes in megakaryocyte cells (Beneytout *et al.*, 1995).

Possible sources of plant material for the isolation of diosgenin are the plants of *Dioscorea* (Cooke, 1970; Mehta and Staba, 1970), *Costus* (Dasgupta and Pandey, 1970) and *Trigonella* (Puri *et al.*, 1976). The advantage of *Trigonella* is that it is an annual plant and so has a short crop cycle (Leung and Foster, 1996). However, very little is known about the process of diosgenin synthesis and its localization in plants (Fazli, 1967).

It is known that development processes in plants are regulated by the action and balance of different plant growth regulators. However, there is little available information on the possible involvement of these compounds in the secondary metabolism of plants, although it has been demonstrated that some may activate or inhibit processes of synthesis and/or accumulation of secondary metabolites of a terpenic nature (Coggins *et al.*, 1969; Wilson *et al.*, 1990; García Puig *et al.*, 1993, 1995; Ortuño *et al.*, 1993).

Bearing in mind that diosgenin belongs to the group of triterpenes, it would be interesting to analyze the role played by growth regulators in the expression of this secondary metabolite. In this respect, the possibility of modulating the processes of diosgenin synthesis and/or accumulation by the use of phytohormones has scarcely been described (Jain and Agrawal, 1988). In the present research we determine the diosgenin content and its evolution in different organs of *Trigonella foenum-graecum*, as well as report the effect of a cytokinin, 6-benzylaminopurine, on the growth and expression of secondary metabolism in this plant material.

MATERIALS AND METHODS

Plant material, hormonal treatment and measurement of growth

Seeds of *Trigonella foenum-graecum* were supplied by Plantafarma (León, Spain). The seeds were imbibed for 24 h in water (to obtain control plants) or in an aqueous solution of 20 ppm 6-benzylaminopurine (to obtain the treated plants). After imbibition, the seeds were germinated in sterile peat and grown in a green-house. Seedlings were harvested 15, 30, 45 and 60 days after germination. To study

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the growth of plants, the evolution of the fresh weight and length of stems, leaves and roots, together with leaf area, were analyzed.

Isolation, chromatographic analysis and identification of diosgenin

For the isolation of dioscin and its subsequent hydrolysis to diosgenin, we followed the method proposed by Sauvaire and Baccou (1978), which was optimized for our work conditions. For this, each of the samples was dried (48 h at 60°C) and powdered. One gram of these samples was hydrolysed with 30 ml of 4N H_2SO_4 in 70% isopropanol for 3 h. The extract was filtered in vacuo and extracted with hexane (60 ml × 3) for 5 min each time. The three hexane extractions were rinsed three times with 5% KOH (180 ml in each case) and then rinsed three times with distilled H_2O (180 ml in each case). The total hexane extract was then passed through a column of Na_2SO_4 to eliminate any remaining water. The samples were concentrated to dryness by evaporating the solvent at 40°C in a Rotovac evaporator. The dried crude extract was solubilized in 0.5 ml of chloroform prior to the quantitative determination of diosgenin by high performance liquid chromatography. The analyses were performed with a Hewlett Packard liquid chrornatograph (model HP 1050) with a diode-array detector (range scanned: 190–500 nm).

Reverse phase chromatographic separation was carried out on a μBondapak C18 (250 × 4.6 mm i.d.) analysis column. The particle size was 5 μm, and isocratic separation was performed using a mixture of acetonitrile:water (90:10; v/v) at a flow of 1 ml min^{-1} at 35°C. Changes in absorbance were recorded in the V/UV diode-array detector at 214 nm. The identity of diosgenin was confirmed by reference to its mass spectrum (Hewlett Packard Mass Spectometer model 5989).

Chemicals

Diosgenin was purchased from Sigma.

RESULTS AND DISCUSSION

Identification and distribution of diosgenin in *Trigonella foenum graecum* plants

In the chromatographic conditions assayed, diosgenin showed a retention time of 12.27 min, similar to that obtained for the diosgenin standard (Sigma). The absorption spectrum of this compound, obtained by means of a UV/V diode array detector, showed one maximum at 200 nm, also similar to that obtained for the diosgenin standard. The mass spectrum obtained for this compound was identical to that obtained for diosgenin by Ghosal *et al.* (1974).

As shown in Fig. 1, the average diosgenin content in *Trigonella foenum-graecum* seeds was 5.65 mg g^{-1} DW. As regards its distribution in 45 day-old plants, the highest content was observed in the leaves (9.56 mg g^{-1} DW), followed by the stems (6.55 mg g^{-1} DW), the fruits (4.30 mg g^{-1} DW) and the roots (2.30 mg g^{-1} DW). The results of this assay show that the leaves of *Trigonella foenum-graecum* are the best source for the isolation of diosgenin for industrial purposes. Curiously, most of the studies concerning the expression of this sapogenin in the plant material under study here have concentrated on root cultures (Zafar and Garg, 1990; Rodríguez-Mendiola *et al.*, 1991) and its expression in seeds (Bohannon *et al.*, 1974). Few mention its expression in leaves (Varshney *et al.*, 1984; Dixit *et al.*, 1985; Taylor *et al.*, 1997).

Based on the above results, the mean level of diosgenin in 45 day old plants of *Trigonella foenum-graecum* is around 0.5%, which is far above that reported by Satish *et al.* (1987) for *Trigonella corniculata* (0.16% in plants of the same age).

Changes in the levels of diosgenin during development of *Trigonella foenum-graecum* plants

When the evolution of diosgenin levels is analysed during the development of several organs of *Trigonella* (Fig. 2), the highest levels are seen to be reached between 15 and 30 days (corresponding to the first stage of development of leaves, data not shown). The highest diosgenin levels were observed at about 30 days (20 mg g^{-1} DW), after which they fell to about 10 and 7 mg g^{-1} DW at 45 and 60 days, respectively. This fall in diosgenin levels between 30 and 45 days cannot be explained by the effect of leaf growth since our studies have shown that there is no change in leaf area between 15 and 60 days. However, there is an increase in leaf

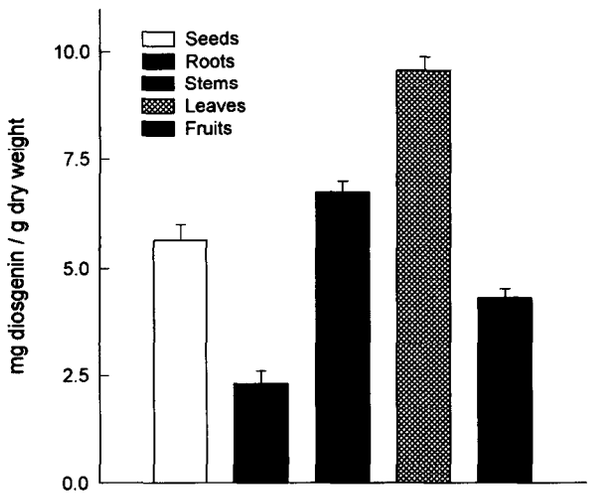

Fig. 1. Diosgenin levels in seeds and several organs of *Trigonella foenum-graecum* at 45 days old. Data represent the mean values of diosgenin (mg g^{-1} dry weight), and the vertical bars denote ± SE ($n = 3$).

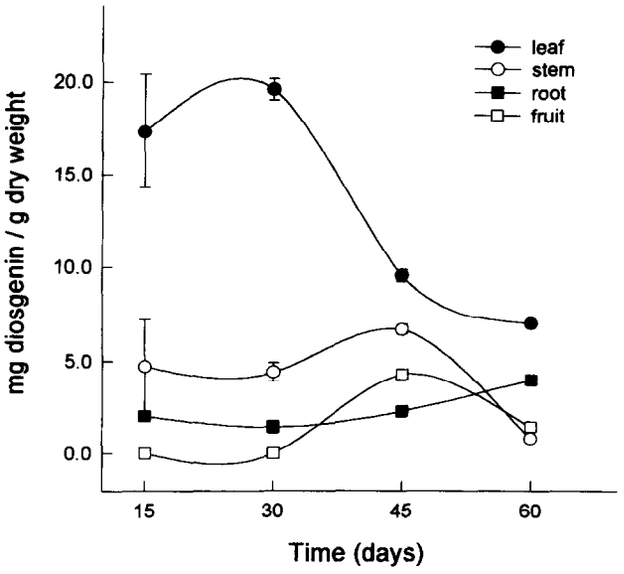

Fig. 2. Evolution of diosgenin levels during the development of plants of *Trigonella foenum-graecum*. At each age, the mean values of diosgenin (mg g^{-1} dry weight) in several organs of plant are represented, and the vertical bars denote ± SE ($n = 3$) when larger than symbols.

succulence after 45 days as observed from their increased fresh weight (data not shown), and so the decrease in diosgenin levels after this time may be due to dilution.

The evolution of diosgenin in the stem and fruits is the opposite to that described for the leaves, since in these organs the levels rise at 30–45 days (from 4.5 to 7 and from 0 to 4 mg g^{-1} DW, respectively). According to these data, the increases observed in the stem and fruit during this interval seem to be due to translocation from the leaves, although other possible metabolic processes should not be ruled out. After 45 days, diosgenin levels fell in both organs while, in roots, diosgenin levels reached 4.5 mg g^{-1} DW at 60 days, when processes of root-ageing would already have begun (data not shown).

The significant positive correlation between diosgenin levels, in the organs studied at different ages, supports the theory of its transport from leaves to root and its accumulation in intermediary organs (stem and fruits). However, the possibility of the 'in situ' synthesis of this secondary compound in the same organs must not be overlooked.

Effect of 6-benzylaminopurine treatment on the diosgenin content in *Trigonella foenum-graecum* plants

The application of 6-benzylaminopurine (20 ppm), in our work conditions did not significantly modify the growth of these plants compared with the control (data not shown). As regards the effect of this phytohormone on diosgenin levels in *Trigonella*, the results point to an increase of 47% in the leaves 15 days after treatment compared with levels in the leaves of the control plants at the same age (Fig. 3). At 30 days, however, the

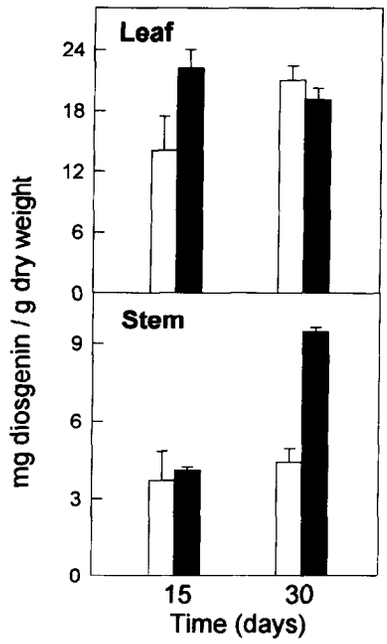

Fig. 3. Effect of 6-benzylaminopurine (20 ppm) on diosgenin levels in plants of *Trigonella foenum-graecum*. At 15 and 30 days, control (□) and treated (■) plants were analyzed. The diosgenin levels (mg g^{-1} dry weight) were determined in leaf and stem. The experiments were carried out in triplicate and the vertical bars denote ± SE ($n = 3$).

stimulatory effect of 6-benzylaminopurine was not observed in leaves but in the stem, where increases of 113% over the control were observed. This may be due to the translocation of diosgenin from leaves to stem although this phytohormone may also stimulate its synthesis in the stem itself. At both times assayed (15 and 30 days), the diosgenin levels in the roots, after treatment with 6-benzylaminopurine, did not differ significantly from those of the control (data not shown).

This stimulation of the synthesis and/or accumulation of diosgenin in the leaves and stems of this plant material caused by the effect of benzylaminopurine becomes evident 30 days after treatment, when the diosgenin levels of treated plants exceeds that of untreated control plants of the same age by 27%.

In summary, the fact that treatment with 6-benzylaminopurine did not markedly affect plant growth suggest that this cytokinin acts on diosgenin expression by affecting its biosynthesis and/or translocation in *Trigonella*.

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