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HORMONAL CONTROL OF STEROID LEVELS IN TISSUE CULTURES FROM *TRIGONELLA FOENUMGRAECUM*

KEITH ROGER BRAIN and GEORGE BRIAN LOCKWOOD*
Welsh School of Pharmacy, Uwist, Cardiff CF1 3NU, Wales

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Key Word Index—*Trigonella foenumgraecum*; Leguminosae; fenugreek; tissue culture; steroid; hormone.

Abstract—The free and bound sterol and sapogenin levels of static and suspension cultures of *Trigonella foenumgraecum* have been investigated. Variations in the levels and distribution of these steroidal compounds with alterations in medium composition and culture age are reported. Synergism between auxin and cytokinin was observed in certain combinations and antagonism in others.

INTRODUCTION

Plant tissue culture has been suggested as a potential source of medicinal agents but a major obstacle in this work is the induction of the expression of biochemical totipotency without the simultaneous expression of morphological totipotency. If plant tissue culture is to be a viable production technique it is necessary to understand the processes controlling the synthesis and accumulation of particular products so that these can be manipulated to best advantage. There have been a number of reports [1–10] of accumulation of sterols and sapogenins in tissue cultures and it has been observed that the levels can vary with the medium composition. However, in general, the data has reported the effects of single levels of single hormones at one particular time. Stohs and Rosenberg [9] have recently reviewed production and metabolism of steroids in plant tissue cultures. This present report is of extensive studies on the effects of variation in medium supplementation on steroid levels in cultures from *Trigonella foenumgraecum*, an annual leguminous herb which has been suggested [11] as a potential commercial source of diosgenin. It was known that the plant produced phytosterols and sapogenins in both free and glycosidically bound forms and that variations in the levels of these compounds in the seed could be produced in the presence of certain plant growth regulators [12].

RESULTS AND DISCUSSION

Static cultures

In the static cultures (Table 1) growth was enhanced by addition of cytokinin in the form of kinetin or coconut water. However, this increase was at the expense of free sterol which was virtually absent in the presence of cytokinin. Bound sterol was very low with all media. Low levels (less than 0.01 mg/culture) of bound diosgenin occurred in the cell extracts, and in the absence of cytokinin a spot with the TLC characteristics of gitogenin appeared, but only in the free form. Significant quantities of steroids were not found in the medium.

Suspension cultures

In young suspension cultures (20 days after subculture) containing 0.1 mg/l. 2,4-D both the free sterol and the bound diosgenin levels were higher than those in the static cultures (Fig. 1). Cellular bound sterol and free diosgenin were below the limit of estimation and only small traces of steroids appeared in the medium. Addition of increasing quantities of kinetin suppressed the diosgenin level, whilst there was a concurrent increase, then decrease, in both cell free sterol and dry wt with cytokinin concentration. Addition of coconut water increased both free sterol and dry wt, particularly at higher concentrations. Low levels of coconut water suppressed the diosgenin level, but this tendency was reversed at high concentrations. In the presence of 1.0 mg/l. 2,4-D both free sterol and dry wt were increased but diosgenin was almost absent. Addition of kinetin suppressed free sterol and dry wt and this was even more marked with coconut water.

Comparison of the dry wt with the cell number and packed cell volume of these cultures (Fig. 2) revealed large variations in these parameters. For example increase in the level of 2,4-D from 0.1 mg/l. to 1.0 mg/l.

Table 1. Sterol levels in static cultures from *Trigonella foenumgraecum*

Medium Supplementation	Dry wt (g)	Sterol/culture (mg) Free	Bound	Total
0.25 mg/l. 2,4-D	1.12	0.56	0.017	0.577
0.25 mg/l. 2,4-D + 0.50 mg/l. kinetin	2.03	0.017	0.012	0.029
0.25 mg/l. 2,4-D + 10% v/v coconut water	3.16	0.012	0.090	0.102

*Present address: Faculty of Pharmacy, University of Ife, Ile-Ife, Nigeria.

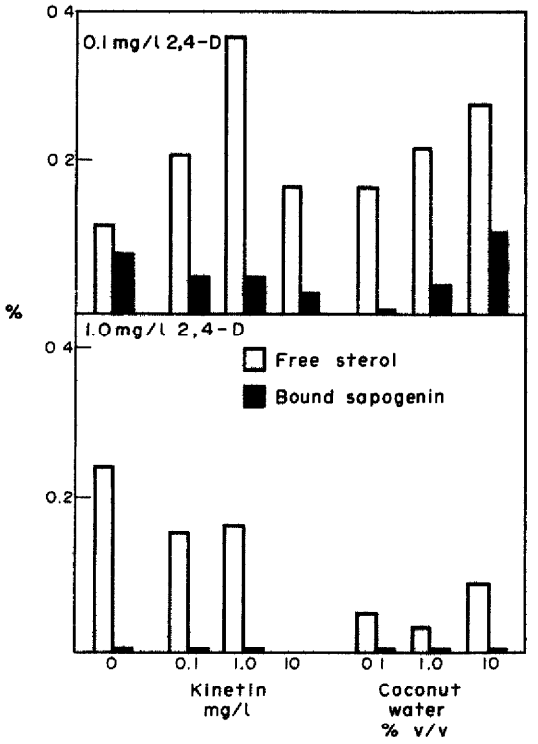

Fig. 1. Sterol and sapogenin levels in 20 day suspension cultures with varying supplementation.

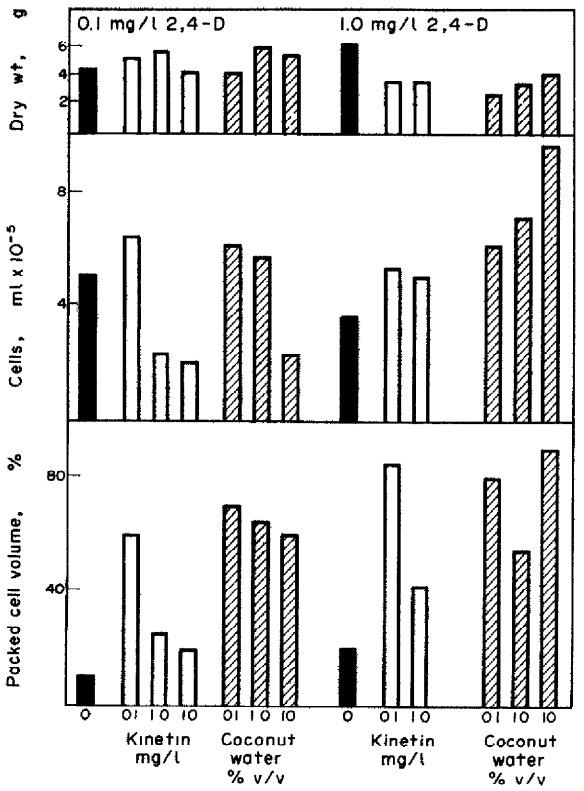

Fig. 2. Comparative growth data on 20 day suspension cultures with varying supplementation.

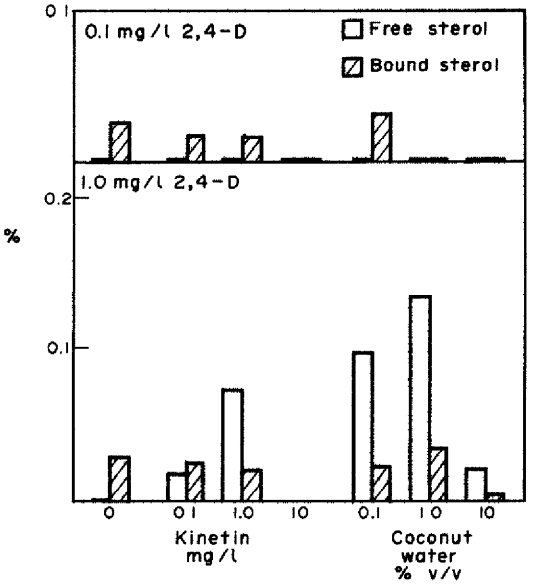

Fig. 3. Sterol levels in 140 day suspension cultures with varying supplementation.

increased the dry wt and packed cell volume but the cell number was reduced, i.e. there was a smaller number of larger and denser cells.

In old suspension cultures (140 days after subculture) once again only traces of steroid were found in the medium (Fig. 3). In addition only traces of diosgenin were found in the cell fraction. In the presence of 0.1 mg/l. 2,4-D, sterol was present almost exclusively in the bound form and the level of this fell with increasing concentration of kinetin or coconut water. On the other hand with 1.0 mg/l. 2,4-D, free sterol was found at high levels when kinetin or coconut water was present.

If coconut water was used as the sole hormone supplement then in both young and old cultures bound sterol and sapogenin were virtually absent but appreciable levels of free sterol were found (Fig. 4).

The steroid levels in the original seeds were : bound sapogenin 0.7%, free sterol 0.045% and bound sterol 0.07%, and thus in all cultures sapogenin level was lower than in the seeds, but in many cultures sterol levels were higher than in the seeds. Kaul and Staba [5] and Kaul *et al.* [6] reported that diosgenin accumulation was almost absent in differentiated *Dioscorea deltoidea* cultures. Differentiation was not observed in any of our cultures and it does not appear to be a requirement for accumulation of steroid.

Khanna and Jain [10] examined production of sterol and sapogenin in static cultures from *T. foenumgraecum* and reported very high levels of sapogenin and a high growth rate. They reported isolation of diosgenin, gitogenin and tigogenin from cells grown on M & S medium supplemented with 1 mg/l. 2,4-D and concluded that the levels were higher than in the initial seeds. Our static cultures from *T. foenumgraecum*, grown on M & S medium supplemented with 0.25 mg/l. 2,4-D, with or without 0.50 mg/l. kinetin or 10% coconut water, did not contain significant amounts of sapogenin. Even when sapogenin was detected in suspension cultures the levels were far below those in the seeds under all experimental

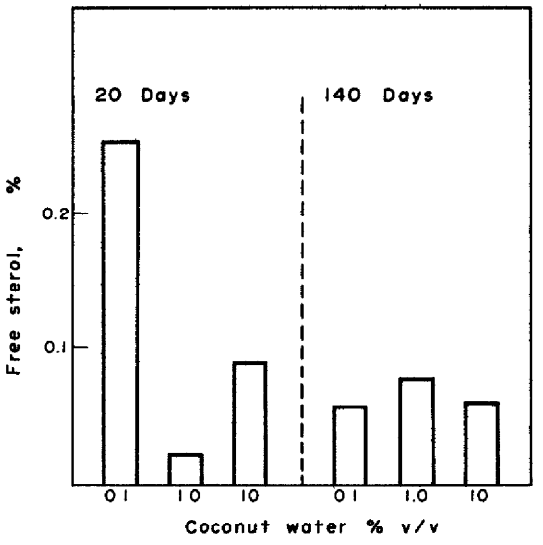

Fig. 4. Influence of coconut water on free sterol levels.

conditions investigated. It has been reported [12] that addition of various hormones to seeds of *T. foenumgraecum* produced variation in the yield of steroidal sapogenin, and the present results reinforce the significance of hormonal control of sterol metabolism. Kaul *et al.* [6] studied diosgenin production in cultures from *Dioscorea deltoidea* and found that 2,4-D and simazine could stimulate accumulation of diosgenin. Heble *et al.* [4] reported that 2,4-D, IAA and IBA had effects on sterol and sapogenin levels in cultures from *Solanum xanthocarpum*.

We have reported briefly [13] on the effects of hormonal supplementation on steroid levels of cultures from *T. foenumgraecum* and Marshall and Staba [8] have recently published data on the effects of a number of hormones on diosgenin production in cultures from *D. deltoidea*. Marshall and Staba [8] concluded that the hormones had only a minor role in the regulation of diosgenin production in these cultures and observed that a younger culture might be more responsive. In contrast to this work on *D. deltoidea* our results on suspension cultures of *T. foenumgraecum* demonstrate that the dose and nature of hormonal supplementation, as well as the culture age, can have very marked effects on steroid levels. In particular certain combinations of auxin and cytokinin have a synergistic effect on steroidal levels, whilst others are antagonistic. The growth and steroid analyses in Figs. 1 and 2 demonstrate that the variation in supplementation affects both steroid level and the manner of growth, but there was no obvious correlation between the two.

It was of note that whilst sapogenin was present at 20 days it was not found at 140 days, whilst the converse was true for bound sterol. Sterols are normally considered to be present as basic components of cell membranes and essential to life, whilst no definite biological function is known for sapogenins, and correlation between sterol and cell number might therefore have been expected. Since sterol and sapogenin are probably produced for differing reasons it is not surprising that there are variations in the manner of their accumulation in response to application of hormones.

EXPERIMENTAL

Materials. The primary cultures were derived from a commercial source of seeds of *Trigonella foenumgraecum* which was authenticated after growing to maturity.

Callus initiation. Seeds were surface sterilised by immersion in 60 volume H_2O_2 solution for 2 min then transferred aseptically to sterile 20 × 2.5 cm tubes containing fluted filter paper and 10 ml H_2O. Germination was better than 99% and the seedlings were transferred after 4 days to the surface of 30 ml of solid medium contained in 100 ml erlenmeyer flasks capped with aluminium foil. This medium was basically that of Murashige and Skoog but was supplemented by the addition of 0.25 mg/l. 2,4-D. Callus formed rapidly and was isolated by repeated subculture on the same medium. Other subcultures were made onto medium containing either 0.50 mg/l. kinetin or 10% v/v coconut water, in addition to the 2,4-D. These cultures were maintained at 25° in continuous light for a period of 40 weeks before examination for steroidal compounds. They were also used as the cell source for the suspension cultures.

Preparation of suspension cultures. After 40 weeks, suspension cultures were prepared from the static cultures by transferring portions of the callus to 250 ml erlenmeyer flasks containing 100 ml of liquid medium. These cultures were agitated continuously on an orbital flat-bed shaker with a 5 cm throw at 70 strokes per minute. The basic medium was supplemented with 24 different combinations of 2,4-D, kinetin, and coconut water over hundredfold concentration ranges. Some of these combinations did not support active growth and were discarded. The remainder were taken through subculture and were examined for steroidal compounds at 20 and 140 days from subculture.

Extraction procedure. In the case of static cultures the cell mass was removed from the medium and lyophilised. Free sterols were extracted by Soxhlet extraction with petrol (40–60°) for 24 hr. The extracted residue was hydrolysed by refluxing with 2 M HCl for 2 hr, neutralised with 10% NH_3 soln, and the residue dried overnight at 60° before re-extraction with petrol to yield the glycosidically bound steroidal material. The suspension cultures were filtered and the cells lyophilised before extraction as for the static cultures. The medium filtrate was extracted by shaking out three times with an equal vol of C_6H_6 to remove free sterol, adjusting to 2 M with HCl, refluxing for 2 hr, and reextracting with C_6H_6 to obtain the bound material.

Quantitation. In each case the quantity of sterol and of sapogenin in the extracts was determined by densitometric TLC [14], standard deviation (1.5%).

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