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INFLUENCE OF HORMONAL SUPPLEMENTATION ON STEROID LEVELS DURING CALLUS INDUCTION FROM SEEDS OF *TRIGONELLA FOENUMGRAECUM*

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

Abstract—Time course studies were carried out on steroid levels during induction of callus from seedlings of *Trigonella foenumgraecum*. There were marked variations in the levels with time and according to the hormonal supplementation of the medium and, whilst sapogenin level always fell below that in the seed, sterol levels tended to be higher.

INTRODUCTION

Previous work has demonstrated that the levels of steroidal compounds in seeds of *Trigonella foenumgraecum* can be altered by treatment with plant growth regulators [1] and that the levels of such compounds in cell cultures derived from *T. foenumgraecum* vary markedly according to the hormonal supplementation of the medium [2]. The levels of sapogenin in the cultures were much lower than those in the seeds, whilst the sterol levels tended to be higher. No work has been published on steroid metabolism during the callus induction phase from seedlings. As links between differentiation and constituent metabolism are expected this study was undertaken to examine the effects of callus induction on steroid levels. Seeds were germinated for six days and then transferred to Murashige and Skoog's medium (M & S) supplemented with various hormonal combinations, as different hormonal supplementation had been shown to have different effects on seeds [1] and on cultures [2], and grown for a further 12 days. Analyses were conducted for free and bound sterol and sapogenin at 2 day intervals.

RESULTS AND DISCUSSION

Dry weight variations during the experimental period were of minor nature. Sapogenin was detected only in glycosidically bound form, whilst sterol was found in both free and bound forms. Considerable variations in steroidal levels occurred on the different substrates. Since dry weight fluctuations were minor, the total yield/culture data was very similar to that presented below on sapogenin level.

On moist filter paper

Sapogenin level dropped rapidly at first (Fig. 1) but recovered to the initial value at day 4, before falling again to a low level. Total free and bound sterol rose progressively to a peak on day 6 before declining, and during this latter period there was a transition from free to bound sterol.

On supplemented M & S medium

In this case the dry weight fluctuated more considerably over the 12 day period (Fig. 2), but of greater interest was the variation in sterol and sapogenin. As with

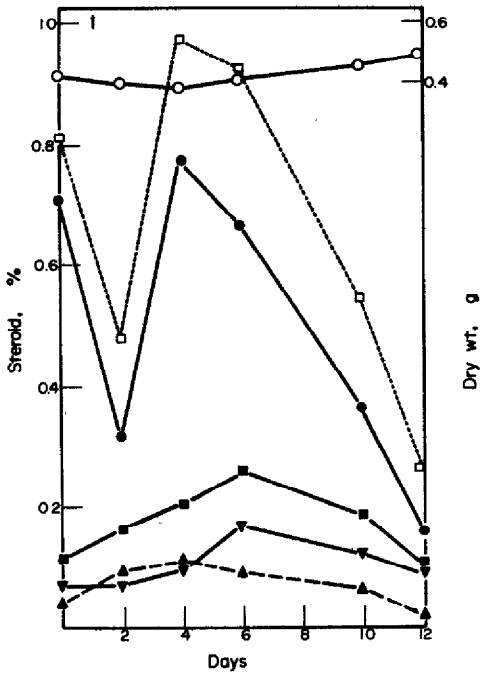

Fig. 1. Steroid levels in seedlings germinating on moist filter paper. O—O dry wt; ▲--▲ free sterol; ▼—▼ bound sterol; ■—■ total sterol; ●—● bound sapogenin; □·····□ total sterol and sapogenin.

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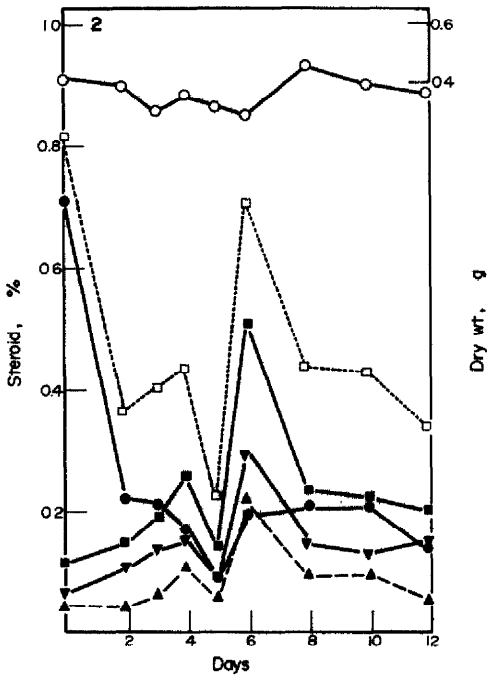
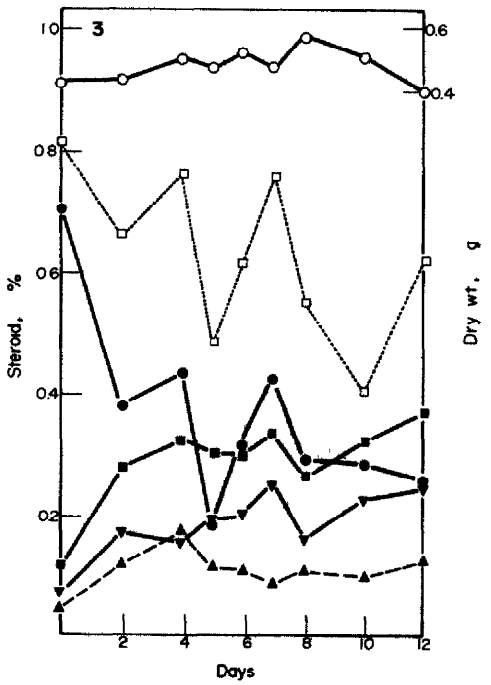


Fig. 2. Steroid levels in seedlings on unsupplemented medium. Legend as in Fig. 1.

seedlings maintained on filter paper the sapogenin level fell sharply on transference, but the secondary peak was delayed and much reduced. On the other hand the total sterol level showed a higher maximum on day 6, which was due to approximately equal rises in both free and bound sterol.



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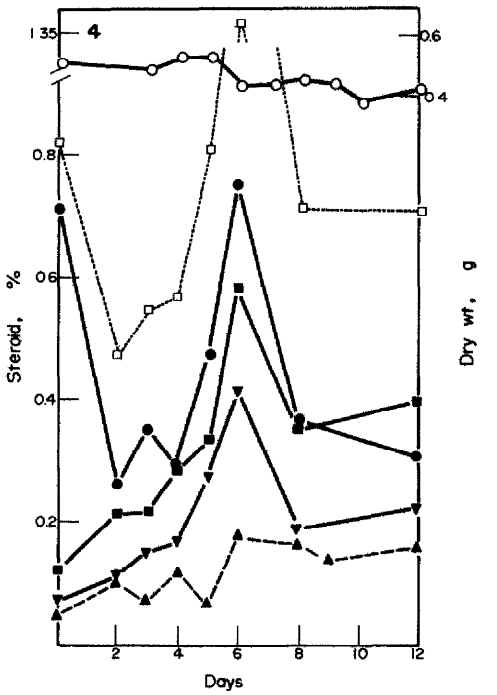

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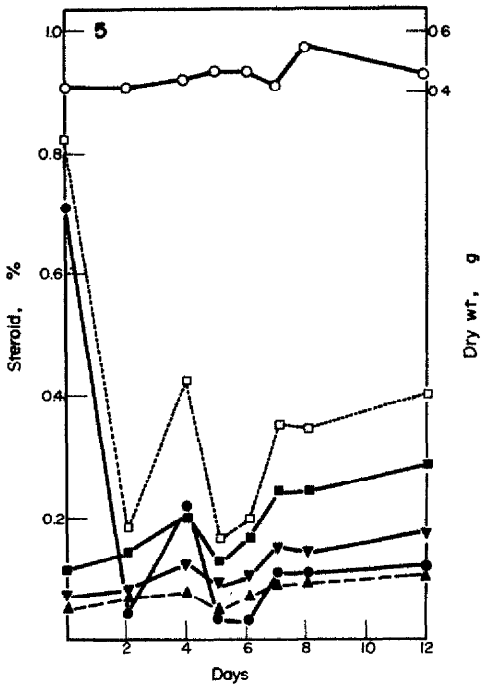


Fig. 5.

Fig. 3–5. Steroid levels in seedlings on medium supplemented with 0.1 mg/l. (Fig. 3), 0.25 mg/l. (Fig. 4), 1.0 mg/l. (Fig. 5) 2,4-D. Legend as in Fig. 1.

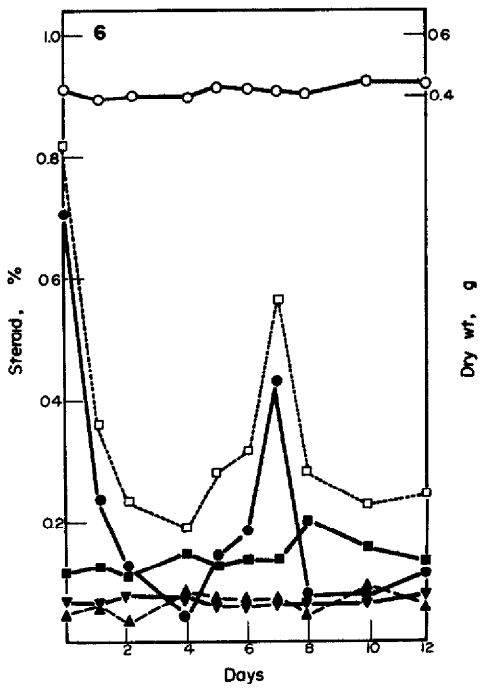
On M & S medium supplemented with 2,4-*D*

2,4-D was incorporated at three concentrations. At the lowest concentration (0.10 mg/l.) there was a slight increase, then a decrease, in dry weight (Fig. 3). The rate of loss of sapogenin on transfer was reduced compared to that with unsupplemented medium and the level

showed considerable fluctuation. Total sterol level rose more smoothly than in unsupplemented medium and was still increasing at the end of the 12 day experimental period. Bound sterol was predominant in the total sterol fraction. When 0.25 mg/l. 2,4-D was incorporated overall dry weight declined slightly (Fig. 4), and sapogenin level fell initially, although this recovered to a sharp maximum, equal to the initial level on transference and the maximum level found on filter paper, on day 6 before falling again. The total sterol level rose much higher than in unsupplemented medium, or in medium with 0.10 mg/l. 2,4-D, although it finally declined. The increase was mainly in bound sterol. The total sterol and sapogenin content of these cultures at day 6 was approximately twice the total level on transference. Incorporation of 1.00 mg/l. 2,4-D gave a slowly increasing dry weight (Fig. 5) but virtually abolished the rises in sapogenin and sterol levels observed at other 2,4-D concentrations.

On M & S medium supplemented with 2,4-D and kinetin

Combination of 0.25 mg/l. kinetin with 0.25 mg/l. 2,4-D (Fig. 6) suppressed the rise in sterol observed with 2,4-D alone, although there was a secondary sapogenin peak which occurred on day 7 rather than day 6. Addition of 0.50 mg/l. kinetin to 0.25 mg/l. 2,4-D (Fig. 7) gave an overall progressive increase in dry weight accompanied by a higher sterol level and an earlier sapogenin peak at day 5. In addition both sterol and sapogenin showed an unusual rise in level immediately after transfer. At the highest concentration of kinetin (1.00 mg/l.) there was little effect on dry weight but a suppression of sterol levels and a delayed secondary sapogenin peak at day 7 (Fig. 8).



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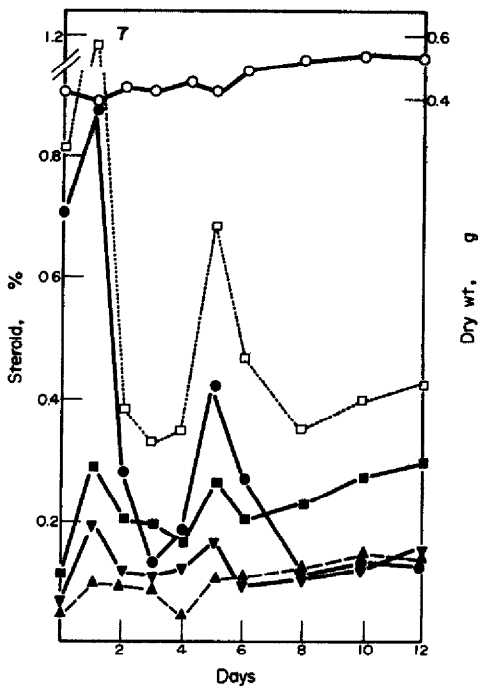

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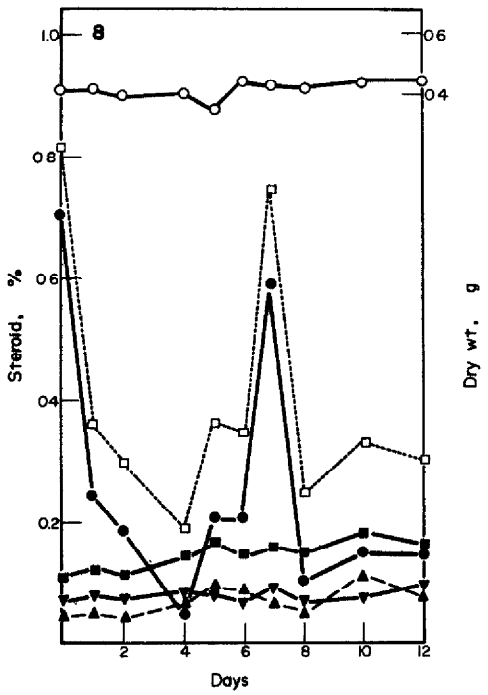


Fig. 8.

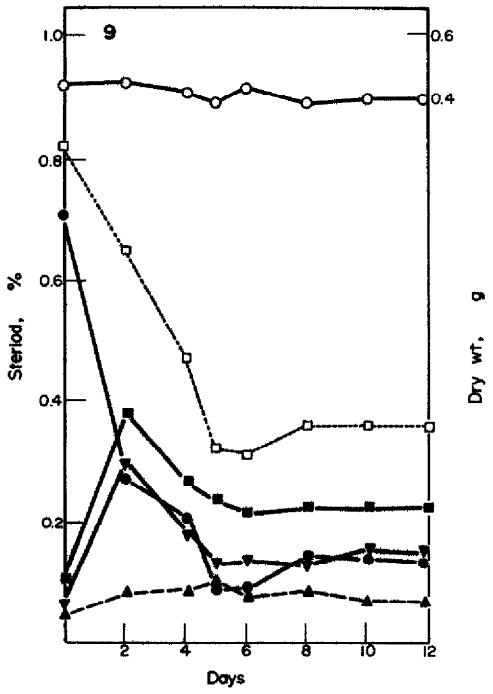
Fig. 6–8. Steroid levels in seedlings on medium supplemented with 0.25 mg/l. 2,4-D and 0.25 mg/l. (Fig. 6), 0.50 mg/l. (Fig. 7), 1.0 mg/l. (Fig. 8) kinetin. Legend as in Fig. 1.

On M & S medium supplemented with IAA

At the lower concentration of IAA (0.10 mg/l.) the most distinctive feature was a marked rise in bound sterol immediately after transfer (Fig. 9). Sapogenin level dropped progressively with no secondary peak. With 1.00 mg/l. IAA (Fig. 10) sterol reached high levels and a secondary sapogenin peak was observed.

Steroid levels in the seedlings at day 12

A comparison of the final sterol and sapogenin levels in representative cultures is given in Fig. 11. Free sterol was barely detectable in the seed and at a low level in seedlings grown on filter paper. Transfer to unsupplemented M & S medium increased the level, and this was further increased by incorporation of growth regulators. The bound sterol level in the seed was reduced after growth on filter paper and raised by transfer to M & S medium, particularly when this was supplemented. In all cases sapogenin levels were much lower than that in the seed and there was little difference between seedlings on filter paper and on unsupplemented M & S medium. Both 2,4-D and IAA increased the final sapogenin level, but this increase was abolished by the addition of kinetin. Total sterol and sapogenin was always lower than in the seed. Growth on M & S medium always gave higher total levels than those with filter paper, and supplementation was favourable, although 2,4-D alone was preferable to combinations of 2,4-D and kinetin.



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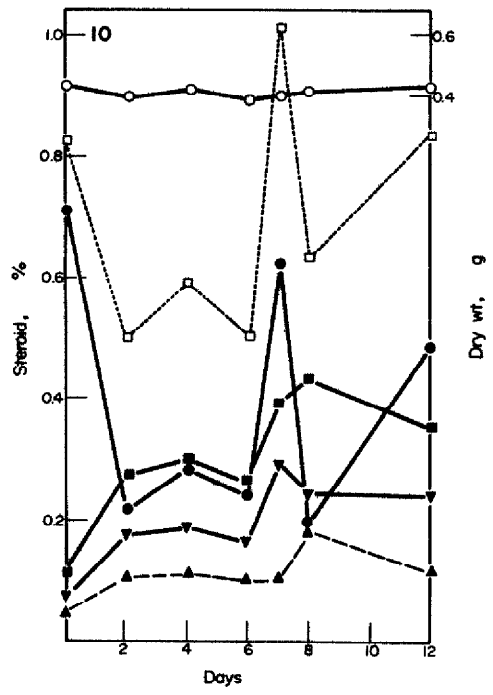

Fig. 10.

Fig. 9–10. Steroid levels in seedlings on medium supplemented with 0.1 mg/l. (Fig. 9) and 1.0 mg/l. (Fig. 10) IAA. Legend as in Fig. 1.

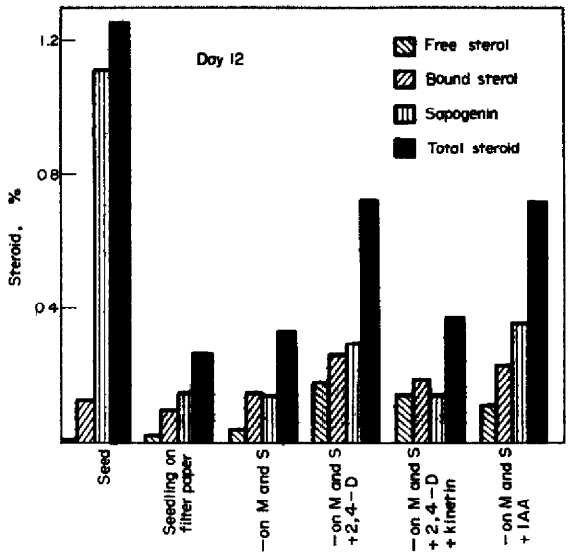

Fig. 11. Steroid levels in seedlings at day 12.

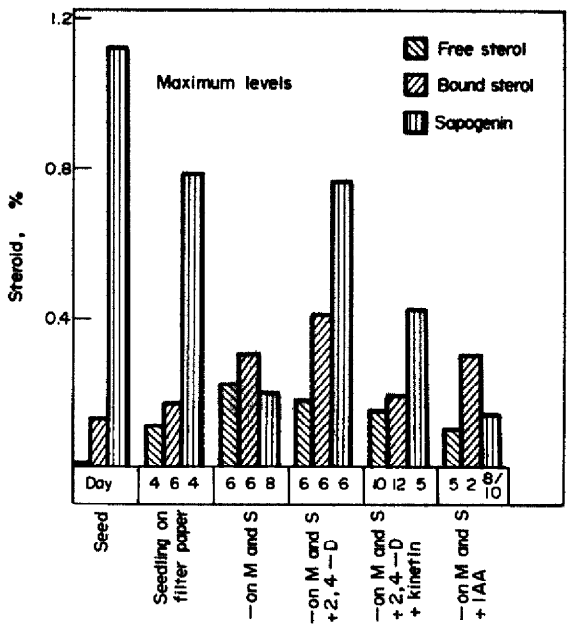

Fig. 12. Maximum steroid levels in seedlings.

Maximum steroid levels

The maximum levels of free sterol, bound sterol, and sapogenin found during the culture period are shown in Fig. 12 together with the day after transfer on which these occurred. In all cases free sterol and bound sterol reached higher levels than were present in the seed, but sapogenin level was always lower. Maximum values for each class of steroid were not normally attained on the same day. These results support earlier suggestions that levels of sterol and sapogenin in seeds are under active control and vary according to the metabolic status of the material. A number of plant growth regulators have been previously shown to be capable of raising sapogenin levels in seeds of *T. foenumgraecum* and in other plant materials [1], and fluctuations in total sapogenin level with differential variations in the proportions of 25α- and 25β-isomers have been found during storage studies on seeds of *T. foenumgraecum* [3]. Dawidar and Fayez [4] estimated sapogenin level in *T. foenumgraecum* during growth from the seed and reported a fall from 1.24% to 0.08% at the end of the first ten days. On the other hand Hardman and Sofowora [5] noted that during germination of seeds of *Balanites aegyptiaca* the sapogenin level dropped in the first day but then increased to reach a level higher than that in the original seeds before falling again. Evans and Cowley [6] reported studies on sapogenin and sterol levels during germination of seeds of *Digitalis purpurea*. The sapogenin level dropped during the first day but rose again to maxima at day 6 and day 24. Upon germination both free and bound sterol decreased during the first day and then dropped very slightly but consistently over the remainder of the period. At the end of 30 days the level of total sterol and sapogenin was below 0.07% as compared with a level of 0.63% in the original seeds.

In the present studies, during normal germination there was an initial fall in sapogenin level but this recovered rapidly before falling again. However if the material was transferred to unsupplemented M & S medium the drop in sapogenin was more marked, whilst the secondary peak was delayed, sharpened, and of lower intensity, and incorporation of growth regulators altered the detailed pattern of steroid level, the precise effect being dependent upon the nature and level of the supplementation. The variations in steroid levels observed may be due to alterations in the synthesis/degradation flux or to release of tightly bound steroid linked to cell wall material [1]. Simple interconversion of sterol and sapogenin does not explain the quantitative variations observed.

Sterols are usually considered to be present as membrane components and Geuns [7–9] has shown that in etiolated mung bean hypocotyls sterol biosynthesis is maximal in the physiologically youngest tissues and that wound surfaces have a higher synthetic capacity than normal tissue. Hartmann and Benveniste [10] found that sterol synthesising capacity in potato discs increased with time after excision and Geuns [9] suggested that this was an expression of dedifferentiation of the cells. He showed that NAA stimulated sterol biosynthesis in both wounded and intact mung bean hypocotyl tissue and suggested that it acted on the rate limiting step of conversion of cycloartenol to sterols. The total sterol content of leaves increases with maturity and it has been suggested [11, 12] that this is a manifestation of senescence and disorganisation of intracellular organelles.

Our results on sterol levels with different treatments show variations in effect. In some cases sterol levels are higher than the untreated control whilst in others they are lower. Under some conditions a peak sterol level is observed whilst under other conditions the sterol level was still rising at the end of the experimental period. The time course of steroid levels under different cultural conditions also demonstrated that marked short term variations could occur in these compounds. At this stage we can only speculate on the reasons for the observed variations in sterol levels. Heftmann [13] has recently reviewed the functions of steroidal compounds in plants and it is clear that they can have basic importance in growth and metabolism. Although sapogenin is often considered as a secondary product with no biological function saponins have been shown to have effects on germination and growth of tissues [14, 15] and the marked variations in level which we have found even during normal germination suggest that the view that they have no biological significance is incorrect.

EXPERIMENTAL

Growth conditions. Samples (0.5 g) of seed of *Trigonella foenumgraecum* were placed in 9 cm petri dishes on moist filter paper and allowed to germinate in the dark at 25° for six days before being divided into three groups. The first group was allowed to continue growth on moist filter paper. The second group was transferred to 9 cm petri dishes containing 20 ml of basic Murashige and Skoogs Revised Tobacco Medium (M & S) containing no growth hormones. The final group was transferred to dishes containing 20 ml of M & S medium supplemented by the addition of growth hormones (0.10 mg/l., 0.25 mg/l., 1.00 mg/l. 2,4-D alone; 0.25 mg/l. 2,4-D with 0.25 mg/l., 0.50 mg/l., 1.00 mg/l. kinetin; 0.10 mg/l., 1.00 mg/l. IAA). All groups were then maintained at 25° in continuous light for a further 12 days.

Extraction and estimation procedures. Triplicate samples from each group were taken at intervals, at the same time each day. After removal from the medium the seedlings were lyophilised and weighed. Free steroidal compounds were extracted from the dried material with petrol (40–60°) in a soxhlet apparatus for 24 hr and the solvent removed on a rotary vacuum evaporator. The residue was taken up in 3 ml $CHCl_3$ containing 0.667 mg/l. lanosterol as internal standard and sterol and sapogenin content estimated by densitometric TLC [16] (Standard deviation <1.5%). Glycosidically bound steroidal compounds were extracted from the marc by refluxing with 50 ml 2 M HCl for 2 hr, neutralised by addition of 10% NH_3 soln, and filtered. The residue was washed with H_2O and dried before soxhlet extraction and steroid estimation.

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