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Phytosterol content of leafy spurge roots and cells from suspension cultures

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Abstract:

Neonate larvae of *Aphthona flava* were fed on either cells obtained from a suspension culture or on intact roots of leafy spurge. Root-fed larvae grew and molted to the 2nd instar within 2 weeks. Cellfed larvae grew equivalently to that of root-fed larvae, but failed to molt and eventually died after 25-30 days of feeding. We are investigating if the failure to molt is a result of qualitative or quantitative deficiencies in the supply of phytosterols in the cells.

The roots contained two major phytosterols, campesterol and Bsitosterol, and minor amounts of stigmasterol and isofucosterol. The cells contain 4 major sterols, 24methylenecholesterol campesterol, β-sitosterol and isofucosterol, and minor amounts of 3 unidentified sterols.

The cells possess an adequate supply of phytosterols to fulfill the sterol-requisites of most phytophagous insects. Further studies are in progress to characterize the sterol metabolism of *Aphthona larvae* and to determine if the cells possess an inhibitor of sterol metabolism.

Molting and sterols

Insects are incapable of the *de novo* biosynthesis of sterols (Bloch, *et al*., 1956). Sterols are required by insects as components of cellular membranes, for reproduction and as precursors for the biosynthesis of molting hormones (viz., ecdysteroids) (Karlson and Hoffmeister, 1963). Most studies to date have shown that phytophagous insects synthesize ecdysteroids from the C27 sterol, cholesterol, through a number of hydroxylating and reducing steps. Cholesterol, in turn, is derived from C28 and C29 phytosterols by dealky-

lation at the C24 position of the side-chain (Figure 1) (Svoboda and Thompson, 1983). These phytosterols must be procured by these insects from their respective host-plants. Conversely, inadequate procurement of phytosterols and/or problems in their bioconversion to ecdysteroids may lead to the abrogation of molting.

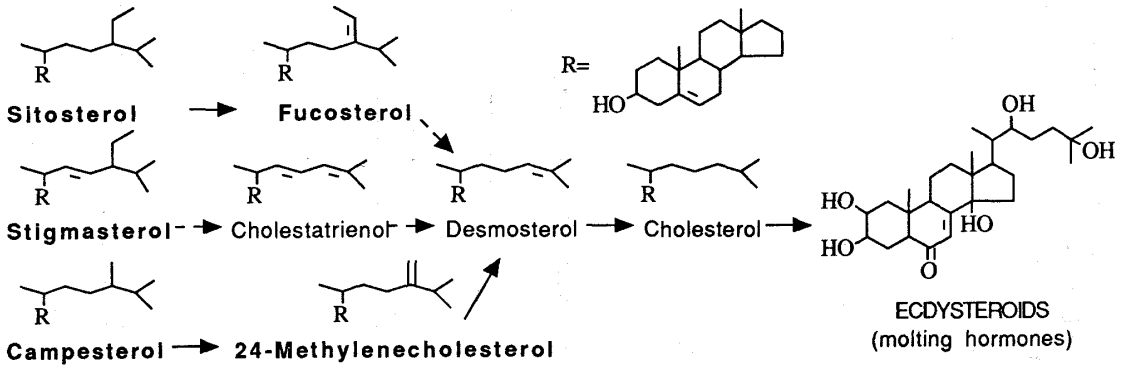

Figure 1. Metabolic pathway used by most insects for the conversion of C_{28} and C_{29} phytosterols (bold print) to cholesterol and eventually to ecdysteroids.

Diet for *Aphthona* larvae

Artificial formulations and roots

The flea beetle, *Aphthona flava,* and its congenetors, show promise as biological control agents of leafy spurge (Maw, 1981). Over the past two years, we have been attempting to formulate an artificial diet for the mass-rearing of these insects. Attempts to rear *Aphthona* larvae on over 10 different diets formulated for various other chrysomelids (Singh, 1976) have all failed (Manners, *et al*., 1988). Addition of various chemical extracts of leafy spurge to these diets to stimulate feeding by *Aphthona* larvae were ineffective (Manners, *et al*., 1988). In general, *Aphthona* larvae failed to feed on any of these diets and the larvae usually died within 1 or 2 days.

We have successfully reared *Aphthona* larvae to the pupal stage on freshly chopped roots of leafy spurge. This was achieved if the roots were either places on filter paper or suspended in agar (Manners, *et al*., 1988). However, this method for the mass-reading of *Aphthona* has inherent drawbacks. Firstly, expansive greenhouse facilities are required to produce enough root material. Secondly, the roots must be harvested and processed. Lastly, in order to find the larvae, the roots must be ground or chopped. Once the roots are ground or chopped, they degrade very quickly, within days, and fresh root material must be replaced continuously.

Suspension culture cells

Over the past year, we have been researching the possibility of mass-reading *Aphthona* larvae on leafy spurge cells grown in a suspension culture (Hogan and Manners,

1989). This approach, if successful, shows great promise mainly from the standpoint that a virtually unlimited supply of diet material will be readily available and simple to harvest.

Unlike the other diet formulations tested, *Aphthona* larvae feed and grow on the suspension culture cells of leafy spurge. Moreover, the larvae can be sustained on these cells for almost a month. Whereas, larvae placed on the other diet formulations die within 1-2 days (Hogan and Manners, 1989).

Although the larvae feed and grow on these cells, they fail to molt to the 2nd instar. The larvae grow from neonates of .1 mm in length to > 3 times that length. Head capsule width, however, remains at ≈160μm throughout this growth period (Figure 2). Examination of hundreds of larvae fed on suspension culture cells failed to show any larvae with a head capsule width equivalent to that of 2nd instar larvae (i.e., ≈320μm) even after 3-4 weeks of growth (Hogan and Manners, 1989). This lack in head capsule width is accentuated by the fact that overall length and size of these larvae exceeded that of 2nd instars.

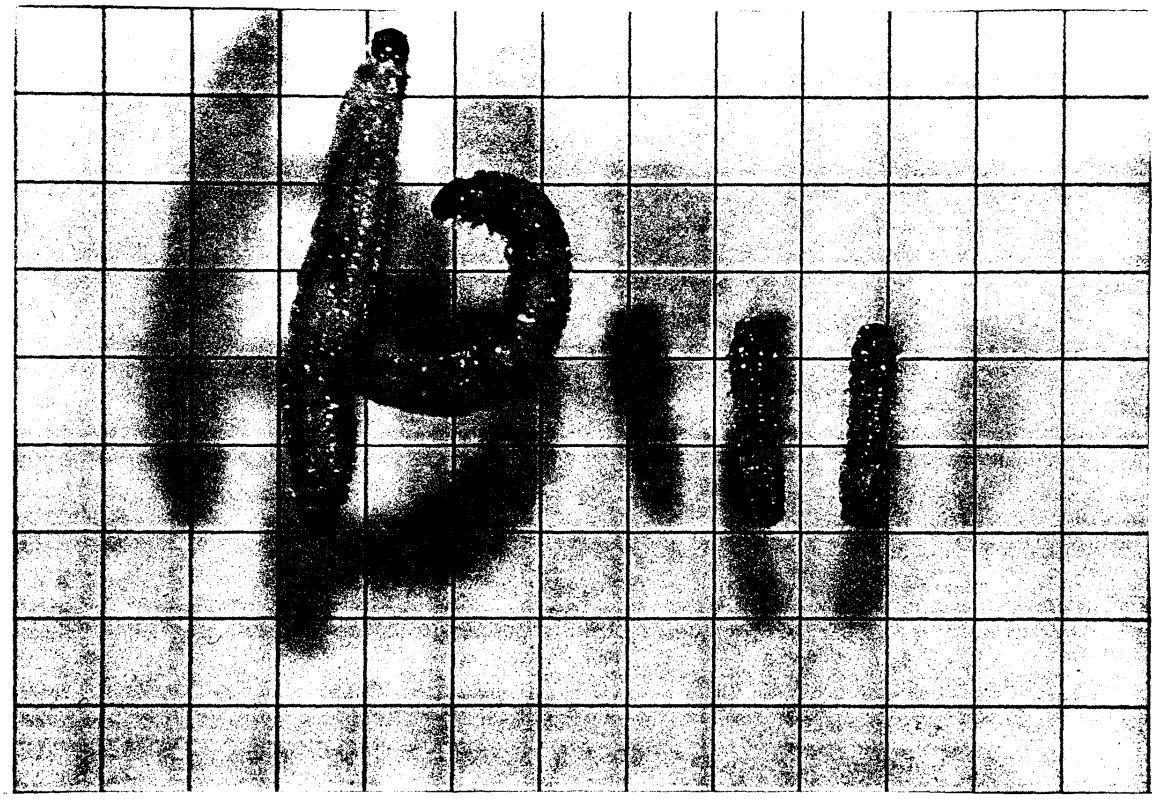

Figure 2. Photograph showing the overall growth of *Aphthona* larvae reared on suspension culture cells. The larvae on the left are 28 days old, those on the right are 8 days old. Note that head capsule width is the same between both groups while overall body length has increase >3X in the 27-day-old group (0.5 mm grid).

Ecdysteroids and molting

We are currently investigating if there are nutritional deficiencies in the suspension culture cells. Nutritional inadequacy of an insect diet may explain the failure of *Aphthona* larvae to molt when fed on these cells. One possibility we are investing is that this failure to molt may involve complications in normal sterol metabolism and/or ecdysteroid biosynthesis.

Most insects synthesize a prohormone form of ecdysteroid in the prothoracic gland. During normal insect development, the synthesis of the prohormone is activated approximately a week prior to molting (Redfern and Bownes, 1985). This synthesis is generally reflected by elevated titers of a more hydroxylated form of the ecdysteroid in the hemolymph 5-6 days before molting (Figure 3). The occurrence of this hormone in the hemolymph triggers a cascade of physiological events that eventually results in ecdysis (Smith, 1985).

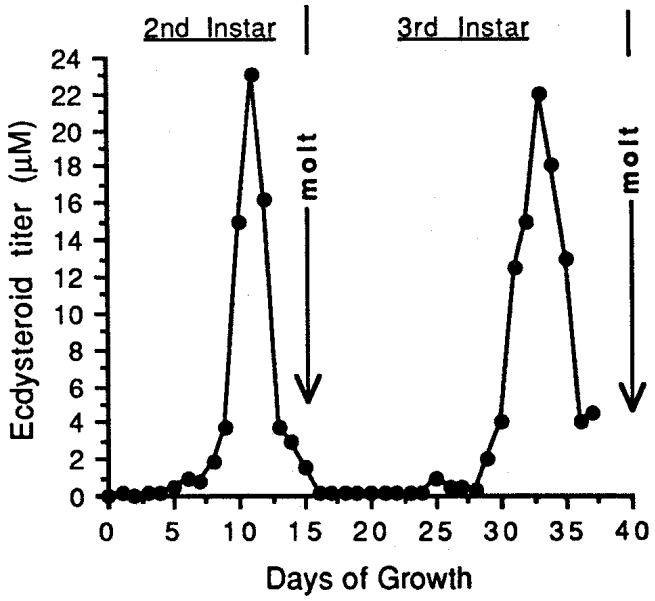

Figure 3. Titer of ecdysteroids in the hemolymph of a representative insect during larval development. Note that peak titers occur 5-6 days before molting (adapted from Smith, 1985).

Phytosterols in leafy spurge

Composition of roots vs. cells

The phytosterol content of leafy spurge cells, grown in suspension culture, was compared to that of roots from greenhouse grown plants. Undifferentiated parenchymal cells of leafy spurge were grown as outlined by Hogan and Manners, 1989. Cells were con-

tinuously harvested, freeze-dried and stored at –20ºC until extraction. The methods used for extraction, partial purification and identification of phytosterols were similar to those described by Campbell and Nes (1983). Briefly, subsamples (≈1 g) of freeze-dried cells or roots were extracted with chloroform by Soxhlet for 4 hours. The extract was dried in *vacuo* and redissolved in a small volume of benzene. A 100 µl aliquot was streaked onto a silica gel TLC plate. The plate was developed in benzene:diethyl ether (9:1; v:v). The region of the TLC plate which cochromatographed with free cholesterol was scraped from the plate, eluted with diethyl ether, filtered and redissolved with 100µl benzene. 5α-cholestane (1µcholestane (1µg/ml) was added as an internal standard.

Sterols were preliminary identified by capillary gas-chromatography. Underivatized samples (0.2µl) were applied to 30m, J + W DB-1 and DB-5 columns (13:1 split). Relative retention times to cholestane of unknown peaks were compared to that of standard sterols (Figure 4). Identity of the sterols was later confirmed by gas chromatography/mass spectroscopy (GC-MS).

Table 1. Relative percent phytosterol composition of leafy spurge cells and roots.

Phytosterol	Relative Percent	
	Cells	Roots
24-Meihylenecholesterol	7	tr*
Campesterol	23	15
Stigmasterol	tr	1
Unknown (500 mw)	3	tr
β-Sitosterol	44	79
Isofucosterol	18	5
Unknown (414 mw)	5	tr

*tr - trace or undetectable amounts

The phytosterol compositions and relative percents of each sterol in the cells and roots are outlined in Table 1. In general, the phytosterol composition of leafy spurge roots was notably simple. Almost 80% of the sterol in the roots consists of the common phytosterol, β-sitosterol. Another common phytosterol, campesterol, was present at 15%. The remaining sterols found in the roots were isofucosterol (5%) and a minor amount of stigmasterol (1%).

The phytosterol profile of the cells, conversely, was markedly more intricate than that of the roots. The roots featured essentially one major sterol, β-sitosterol. The cells, however, possessed 3 major phytosterols. These were β-sitosterol (44%) and 2 other prominent phytosterol, campesterol (23%) and isofucosterol (18%). In addition to these, the cells also contained 3 minor sterols that were either absent or present in trace quantities in the roots. These minor sterols included 24-methylene cholesterol and two other unknown sterols. The structures of these unknown sterols are being determined. They have molecular weights of 400D and 414D according to molecular ions detected by mass-spectroscopy.

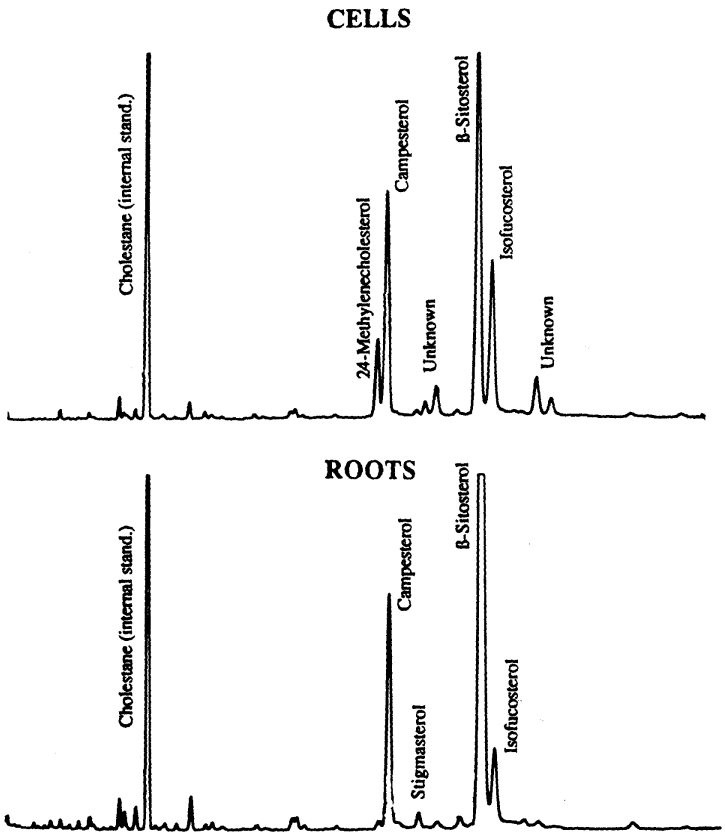

Figure 4. Gas chromatograms of phytosterols in leafy spurge cells (top) and roots (bottom). Sterols were identified by GC-MS. Cholestane was used as the internal standard.

Impact on growth of *Aphthona* larvae

The phytosterol composition of intact leafy spurge roots compared to that of cells grown in suspension culture is obviously different. Does this different composition of phytosterols in the cells account for the lack of molting by *Aphthona* larvae fed on these cells? Without the results of metabolic studies, currently in progress, no certain answer can be provided.

However, the major phytosterols identified in the cells (i.e., β-sitosterol, campesterol, isofuco sterol and 24-methylenecholesterol) can generally be converted to cholesterol by insects (Figure 1). It is also likely that *Aphthona* larvae can dealkylate these phytosterols and convert them to cholesterol. Otherwise, how would these larvae be able to survive on leafy spurge roots whose chief sterol is β-sitosterol?

The possibility exists that one or more of the unknown sterols in the cells, or perhaps some other components, are inhibiting sterol metabolism of the larvae. The dealkylation of phytosterols to desmosterol is necessary for their bioconversion to cholesterol by insects. Moreover, there are a number of known substances, both manmade and natural,

which inhibit the ability of insects to convert desmosterol to cholesterol (Svoboda, 1984). However, the prospect that sterol metabolism of *Aphthona* is disrupted by some component in the cells can only be speculated at this time.

Currently, we are attempting to answer some of the questions associated with sterol metabolism and lack of molting by *Aphthona* larvae fed on cells. Some of the experiments which are currently in progress include: 1) rearing larvae through one instar on roots and transferring them onto cells (the larvae could procure any essential precursors required for molting from the roots), 2) treating larvae and cells with 20-hydroxyecdysone in an effort to trigger molting, and 3) examining the sterol compositions of *Aphthona* larvae fed on roots and cells to determine if the amount of cholesterol in these larvae is different (this would indicate if a component in the cells is disrupting sterol metabolism).

There is a possibility that a nutritional component other than sterols is affecting larval growth of *Aphthona* fed on the cells (e.g., certain essential fatty acids). We will pursue this possibility if our current research on sterols does not ascertain the basis for the lack of larval molting.

Literature cited

Bloch, K., R. G. Langdon, A. J. Clark, and G. Fraenkel. 1956. Impaired steroid biogenesis in insect larvae. Biochem. Biophys. Acta 21:176.

Campbell, B. C. and W. D. Nes. 1983. A reappraisal of sterol biosynthesis and metabolism in aphids. J. Insect Physiol. 29:149.

Hogan, M. E. and G. D. Manners. 1989. Leafy spurge cell cultures as the base of an artificial diet for *Aphthona flava*. Proc. Leafy Spurge Symp., Bozeman, MT 1989.

Karlson, P. and H. Hoffmeister. 1963. *Zue Biogenese des Ecdysons, I. Umwandlung von Cholesterin in* Ecdyson. Z. Physiol Chem. 331:298.

Manners, G. D., M. E. Hogan, B.C. Campbell, and R.A. Flath. 1988. Leafy spurge research at WRRC/ARS. Proc. Leafy Spurge Symp,, Fargo, ND 1988.

Maw, E. 1981. Biology of some *Aphthona* sp. (Coleoptera: Chrysomelidae). Feeding on Euphorbiaceae with special reference to leafy spurge (*Euphorbia* sp. near *esula*). M.S. Thesis, Univ. of Alberta, Edmonton, Canada. 258 pp.

Redfern, C. P. F. and M. Bownes. 1985. Prothoracic glands and the production of steroid hormones during the metamorphosis of holometabolous insects. pp. 181-197. *In* Metamorphosis (M. Balls and M. Bownes, eds), Clarendon Press, Oxford.

Singh, P. 1976. Artificial diets for insects, mites and spiders. IFI/Plenum, N.Y. pp. 49-56.

Smith, S. L. 1985. Regulation of ecdysteroid titer: synthesis, pp. 295-342. *In* Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol 7 (G. A. Kerkut and L. I. Gilbert, eds.), Pergamon Press, Oxford.

Svoboda, J. A. and M. J. Thompson. 1983. Comparative sterol metabolism in insects, pp. 1-16. *In* Metabolic Aspects of Lipid Nutrition in Insects. (T. E. Mittler and R. H. Dadd, eds.), Westview Press, Boulder, CO.

Svoboda, J. A. 1984. Insect steroids: metabolism and function. pp. 367-388. *In* Isopentenoids in Plants: Biochemistry and Function. (W. D. Nes, G. Fuller and L. S. Tsai, eds.), Marcell Dekker, Inc., New York.