Phytochemistry, 1973, Vol. 12, pp. 103 to 106. Pergamon Press. Printed in England.

VARIATIONS IN STEROL COMPOSITION IN ETIOLATED MUNG BEAN SEEDLINGS

J. M. C. GEUNS

Laboratorium voor Plantenfysiologie, Vaartstraat 24, 3000 Leuven, Belgium

(*Revised Received* 1 *June* 1972. *Accepted* 15 *August* 1972)

Key Word Index—*Phaseolus aureus*; Papilionaceae; sterols; sitosterol–stigmasterol ratio; distribution; biosynthesis.

Abstract—The sterol fractions of different morphological and physiological parts of etiolated Mung bean seedlings have been studied. Young growing tissues contained more sterols than older tissues; younger tissues were characterized by a high sitosterol–stigmasterol ratio. With increasing age of the tissues, this ratio gradually decreased. The occurrence of Δ^7-avenasterol and 28-isofucosterol, together with results of incorporation experiments with [2-^{14}C]mevalonic acid indicate that sterol synthesis occurs in the young growing tissues.

INTRODUCTION

IN RECENT years, changes in the level, composition and synthesis of sterols in plants following germination have been investigated.[1–8] These authors usually followed the level and composition of the sterols with time after germination in whole shoots and roots, and so they always studied mixtures of young, growing and older, non-growing tissues. The sterol composition in the different morphological and physiological regions of the plant were never examined. We have studied variations in the level and composition of the sterols in thirteen fractions of 5-day-old etiolated Mung bean seedlings.

RESULTS AND DISCUSSION

Table 1 shows that the total sterol fraction as a percentage of the dry wt of the different plant parts in young, growing tissues is much higher than in older non-growing ones. In the cotyledons, which still function as reserve organs, there is a relatively low concentration of sterols. These low sterol levels are also found in the third–sixth cm fractions of the hypocotyls. The growing, folded, but not fully expanded leaves contain a higher percentage of sterols. Very high sterol concentrations are found in the stem tip, the curve and the first and second cm of the hypocotyl which are also the most actively growing tissues. The stem tip consists mainly of meristematic tissues, the curve of the hypocotyl is partly meristematic and the first and second cm fractions comprise the elongation zone.

Analogous results were obtained with the roots: the older regions contain a lower percentage of sterols than the younger ones.

[1] P. DUPERON and R. DUPERON, *Compt. Rend.* **261**, 3459 (1965).
[2] P. DUPERON and M. RENAUD, *C.R. Soc. Biol.* **160(II)**, 1710 (1966).
[3] R. D. BENNETT, E. R. LIEBER and E. HEFTMANN, *Plant Physiol.* **42**, 973 (1967).
[4] P. DUPERON, H. DELAGE and R. DUPERON, *C.R. Soc. Biol.* **161(II)**, 1792 (1967).
[5] P. DUPERON, *Physiol. Vég.* **9 (3)**, 373 (1971).
[6] R. J. KEMP, L. J. GOAD and E. I. MERCER, *Biochem. J.* **103**, 53 P (1967).
[7] R. J. KEMP, L. J. GOAD and E. I. MERCER, *Phytochem.* **6**, 1609 (1967).
[8] D. S. INGRAM, B. A. KNIGHTS, I. J. MCEVOY and P. MCKAY, *Phytochem.* **7**, 1241 (1968).

TABLE 1. STEROL CONTENT OF MUNG BEAN SEEDLING FRACTIONS

Fraction	Fresh weight (g)	Dry weight (g)	Total non-saponifiables (mg)	Total sterol fraction (mg)	(mg/100 g dry wt)
Cotyledons	8·63	3·45	26	1·21	35
Leaves	1·67	0·41	17	0·96	234
Stem-tips	0·37	0·09	4	0·54	600
Curve	1·38	0·26	7	0·43	165
1 cm	5·37	0·59	10	0·83	140
2 cm	6·78	0·60	8	0·93	155
3 cm	7·75	0·66	8	0·28	42
4 cm	9·18	0·74	8	0·34	45
5 cm	10·81	0·84	10	0·30	35
6 cm	11·05	0·82	10	0·29	35
Roots 1	5·94	0·52	7	0·19	36
Roots 2	1·42	0·17	4	0·17	100
Roots 3	0·90	0·17	4	0·19	112

The dry wts are calculated by dividing the fr. wts by a factor (fr. wt/dry wt) for each plant part, obtained in other experiments. Results are the mean values of 3 experiments. The wt of the total sterol fraction was determined by GLC analyses.

Incorporation experiments with [2-^{14}C]mevalonic acid (Table 2) showed considerable incorporation into sterols in the first cm and poor incorporation into sterols in the third and fifth cm of the hypocotyl, where radioactivity is found in components with R_f values

TABLE 2. RADIOACTIVITY RECOVERED IN THE ELUATES OF 12 ZONES OF TLC OF THE NON-SAPONIFIABLE FRACTION OF HYPOCOTYL EXTRACTS

Zone	R_f eluted	Radioactivity recovered (cpm) 1 cm	3 cm	5 cm	(% of total) 1 cm	3 cm	5 cm
1	0·00–0·06	12170	7220	7930	16	10	9
2	0·06–0·20	4080	2900	2830	5	4	3
3	0·20–0·26 (sterols)	22230	11060	6830	30	16	8
4	0·26–0·34	2960	3410	3270	4	5	4
5	0·34–0·39 (cycloartenol)	19050	17350	18300	26	25	22
6	0·39–0·45	1080	900	1050	1	1	1
7	0·45–0·51 (γ-tocopherol)	3560	4640	6050	4	6	7
8	0·51–0·59	1790	1880	2090	2	2	2
9	0·59–0·67 (α-tocopherol)	2380	15080	26310	3	22	32
10	0·67–0·76	1690	2590	3810	2	3	4
11	0·76–0·85 (squalene)	1140	600	2690	1	—	3
12	0·85–1·00	20	30	100	—	—	—
	Totals	72150	67660	81260			

The medium before each incorporation experiment contained 3 μM [2-^{14}C]MVA (748 × 10^3 cpm). After the incubation the medium of the first cm contained 375 × 10^3 cpm, that of the third cm 384 × 10^3 cpm and that of the fifth cm 371 × 10^3 cpm. Results are the mean values of three experiments.

close to that of α-tocopherol. These results, together with the occurrence of 28-isofucosterol, a precursor of sitosterol synthesis,[9] and the high sitosterol content (Table 3) of growing tissues, suggest an active sterol synthesis during growth processes.

The occurrence of Δ^7-avenasterol in growing tissues and not in mature tissues (5–6 cm hypocotyl fractions, root fractions 1 and 2, Table 3) is in agreement with the hypothesis of the conversion of Δ^7-sterols into Δ^5-sterols[10–12] and with our hypothesis that sterol synthesis occurs in growing tissues.

TABLE 3. PERCENTAGES OF EACH STEROL IN THE STEROL MIXTURES OF THE DIFFERENT PLANT FRACTIONS

Compound	Cotyledons	Leaves	Stem-tips	Curve	Hypocotyl 1 cm	2 cm	3 cm	4 cm	5 cm	6 cm	Roots 1	2	3
Cholesterol	2·9	4·2	12·4	6·7	3·9	8·5	8·8	9·4	8·7	4·8	4·8	6·0	6·0
Unidentified component	0·3	0·03	0·6	2·5	1·1	1·9	1·0	—	—	—	—	—	—
Campesterol	4·4	6·2	11·0	8·9	10·6	10·2	9·7	10·2	11·2	13·4	14·3	11·7	10·3
Stigmasterol	29·9	27·0	15·0	10·1	12·9	18·4	24·9	29·3	33·7	41·1	46·6	45·8	32·3
Sitosterol	55·0	57·2	49·3	62·0	61·6	50·7	49·4	48·2	46·0	40·0	32·8	35·5	45·8
28-Isofucosterol	3·4	1·9	4·3	5·4	7·7	8·6	4·6	1·1	—	—	0·8	0·4	4·6
Unidentified component	2·1	0·5	2·0	0·1	0·1	—	—	0·4	0·1	0·2	0·2	0·3	—
Δ^7-Avenasterol	1·4	2·6	4·6	3·8	1·6	1·3	1·2	0·8	—	0·1	—	—	0·6

Results are the mean values of three experiments.

Table 3 shows the sterol composition of the thirteen fractions. There is a gradual decrease in the percentage of sitosterol and an increase in the percentage of stigmasterol from young growing tissues (stem tip, curve, first hypocotyl fraction) to the older non-growing tissues (2–6 cm fractions). The older parts of the roots (root fraction 1) contain more stigmasterol than sitosterol. From the older root tissues to the younger ones (root fractions 2 and 3), the sitosterol/stigmasterol ratio increases. In the 3–6 cm hypocotyl fractions the level of sterols per mg dry wt is constant and there is no or very little sterol synthesis (Tables 1 and 2). Therefore it can be concluded that the gradual increase in the percentage of stigmasterol and the decrease in the percentage of sitosterol are due to a conversion of sitosterol to stigmasterol,[10,13–15] and that this conversion is taking place in ageing tissues.

The work of Kemp *et al.*[6,7] and of Davis[16] gives further evidence for our hypothesis. Kemp *et al.* divided maize seedlings into shoots, roots, scutellum and endosperm. They found a low sitosterol/stigmasterol ratio in roots. Roots possess a small meristem and a very short zone of elongation growth. The effect of the younger, growing root tissues is lost in the bulk of the older root tissues. In this older root tissue, we, too, found an increase in stigmasterol and a decrease in sitosterol, stigmasterol being the most abundant sterol. In the shoots, however, there is a larger meristem and a much longer zone of elongation growth, so that the sterols of these parts interfere with sterols extracted from the older shoot parts.

Davis[16] found a high sitosterol/stigmasterol ratio in the upper parts and a low ratio in the basal parts of tobacco plants. The changes in relative amounts of these two sterols

[9] D. J. BAISTED, *Phytochem.* **8**, 1697 (1969).
[10] Z. KASPRZYK and Z. WOJCIECHOWSKI, *Phytochem.* **8**, 1921 (1969).
[11] M. DEVYS and M. BARBIER, *Bull. Soc. Chim. Biol.* **49**, 865 (1967).
[12] M. DEVYS, A. ALCAIDE and M. BARBIER, *Bull. Soc. Chim. Biol.* **50**, 1751 (1968).
[13] A. ALCAIDE, M. DEVYS and M. BARBIER, *Phytochem.* **9**, 1553 (1970).
[14] R. D. BENNETT and E. HEFTMANN, *Steroids* **14**, 403 (1969).
[15] F. F. KNAPP and H. J. NICHOLAS, *Phytochem.* **10**, 85 (1971).
[16] D. L. DAVIS, *Phytochem.* **11**, 489 (1972).

could indicate an interconversion between them. Whereas Davis considered this conversion to be influenced by light, we find conversion of sitosterol into stigmasterol in ageing tissues of etiolated plants.

The results presented may indicate some active role of sitosterol or its derivate(s) in growth processes, after which it is converted to stigmasterol.

EXPERIMENTAL

Plant material. For each experiment, 200 5-day-old etiolated Mung bean seedlings (*Phaseolus aureus* Roxb.) with a hypocotyl length of 6 cm were selected. These plants were divided in orange light into thirteen fractions: the cotyledons, which are reserve organs, the not yet fully developed and still growing leaves, the very small stem tips which consist mainly of meristematic tissues, the curve of the hypocotyl (partly meristematic tissue), the first cm of the hypocotyl (the zone of elongation growth) cut off just below the curve of the hypocotyl, the second–sixth cm (five fractions which represent a gradual change of differentiating into mature hypocotyl tissues). The root was cut into 3 fractions of equal length: roots 1, the oldest root tisses, roots 3, consisting of the very young root tissues with the apical root meristem and the zone of elongation growth and roots 2, of intermediate age.

Sterol extraction and analysis. For the total sterol extraction, the different plant parts were homogenized in MeOH, saponified for 90 min at 65° in 60 ml 8% KOH in 80% MeOH after 0·5 g pyrogallol had been added. After addition of an equal volume of water, the non-saponifiable material was extracted 4 × with freshly distilled Et_2O. After evaporation of the Et_2O, the residue was spotted on TLC plates (0·5 mm, silica gel G Merck) using $CHCl_3$ for development and sitosterol as a reference for the sterol band. After drying, the plates were sprayed with Rhodamine 6 G (Merck) and studied under UV for characteristic fluorescence. The sterol band was scraped off and eluted with Et_2O. The Et_2O was sucked over aluminium oxide (Woelm neutral) in a glass filter to adsorb the Rhodamine. The Et_2O was blown dry under N_2 and the residue (total sterol fraction) was further analysed by GLC on 3% OV-17 columns at 270°. The temps. of the detector and of the injection port were 250 and 285° respectively. The column length was 1·83 m with a diameter of 4 mm. The carrier gas flow (N_2) was 40 ml/min. Six components of the sterol mixtures were identified as cholesterol, campesterol, stigmasterol, sitosterol, 28-isofucosterol and Δ^7-avenasterol by co-chromatography with very pure sterol samples on 3% OV-1 and 3% OV-17 columns. The 4 major components (cholesterol, campesterol, stigmasterol and sitosterol) were identified by GC–MS.[17,18] The MS-data for these sterols were: cholesterol (386, 371, 368, 353, 301, 275, 247, 273, 255, 231, 213, 246, 229), campesterol (400, 385, 382, 367, 315, 289, 261, 273, 255, 231, 213, 246, 229), stigmasterol (412, 397, 394, 379, 369, 351, 273, 255, 231, 213, 229, 253, 300, 211), sitosterol (414, 399, 396, 381, 329, 303, 275, 273, 255, 231, 213, 246, 229). These 6 sterols also were found in Mung beans by Dr. F. F. Knapp (Dept. of Biochemistry, Univ. of Liverpool, personal communication). On a 3% OV-17 column, all these sterols are separated. The percentage of each sterol in the total sterol fraction of each plant part (Table 3) was calculated by measuring the peak areas of the GLC-chromatograms.

Incorporation experiments. For each experiment 75 etiolated plants were fractionated in orange light. The different sections were incubated in 15 ml [2-^{14}C]mevalonic acid solution (3 μM) (dark, 23°) for 20 h and continuously shaken. Streptomycin (10 mg/l.) was added. After the incubation period, the sections were rinsed several times with distilled H_2O and the vol. made up to 50 ml. Samples of the medium after the incubation period were counted in a liquid scintillator with an efficiency of 75% in a H_2O/toluene–Triton solution (1:3·4), the toluene-Triton solution consisting of 4 g and 500 ml Triton ×100/1000 ml toluene. Samples of the non-saponifiable material were spotted on TLC plates together with the following standards: sitosterol, cycloartenol, γ-tocopherol, α-tocopherol and squalene. The plates were scanned for radioactivity, sprayed with 0·05% fluoresceïn in EtOH and studied under UV. Finally the plates were divided into 12 zones (Table 2), scraped off and the eluates counted in the liquid scintillator by the external standard method.

Acknowledgements—The author would like to thank Professors K. Buffel and J. C. Vendrig and the people of the laboratory for their interest in the work and for helpful criticism of the manuscript, and Dr. J. Goad for a gift of pure sterol standards and samples of cycloartenol. Thanks too go to C. Wellens and L. Van Coillie, to J. Demin and A. Swinnen for cutting plant material and for technical assistance. This research was supported by funds from the N.F.W.O.

[17] B. A. KNIGHTS, *J. Gen. Chem.* **5**, 273 (1967).
[18] B. A. KNIGHTS and C. J. W. BROOKS, *Phytochem.* **8**, 463 (1969).