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CHANGES IN STEROL CONTENT DURING LEAF AGING AND IN VITRO DIFFERENTIATION IN *SOLANUM NIGRUM*

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ABSTRACT.—Composition of β-C-3 sterols of *Solanum nigrum* has been studied during leaf aging and in vitro morphogenesis. Cholesterol, campesterol, stigmasterol, and sitosterol were identified in the leaves and in the differentiated and undifferentiated tissue cultures. The content of cholesterol declined during leaf aging. The ratio of stigmasterol to sitosterol was low (0.1) in the young leaves, but increased to 0.16 in the mature leaves. Total sterol content in the in-vitro differentiated shoots was about 1.5 times higher than the initial leaf explant. Among the tissue cultures, sterols were about three times higher in the differentiated cultures (roots and shoots) than in the undifferentiated cultures (callus). Stigmasterol to sitosterol ratio was about three times higher in the callus grown on benzyladenine- containing medium than that grown on indoleacetic-acid-containing medium.

Plants contain many different types of β-C-3 sterols of which sitosterol, stigmasterol, and campesterol are the most common (1). Even though the biosynthetic pathway for most sterols is well defined, the function and necessity of multiple sterols is less certain (1, 2). Knowledge of factors that affect the sterol composition of plant cells could provide a clue to the function of sterols in plants. With this objective, we describe here two developmental factors that change the sterol composition in *Solanum nigrum* L.: leaf aging and in vitro morphogenesis of roots and shoots from leaf explants.

MATERIALS AND METHODS

DETERMINATION OF β-C-3 STEROLS BY GAS CHROMATOGRAPHY.—Lipids were extracted twice from three independent samples randomly pooled, using MeOH-$CHCl_3$ (2:1) containing 50 mg/liter butylated hydroxytoluene at 0-4° (3). 5α-Cholestane, used as an internal standard, was added at the initial step of extraction. The solvents were evaporated by N_2, and the lipids were immediately dissolved in 2 ml of Me_2CO-EtOH (1:1) to which 1 ml of a 2% digitonin solution (in 50% EtOH) was added. The mixture was left overnight in a sealed KIMAX conical tube at room temperature. Under these conditions, digitonin forms insoluble complexes (digitonides) with sterols. The digitonides were pelleted by centrifugation at 10,000 g for 12 min, washed with 2 ml of Me_2CO-anhydrous Et_2O (1:2), and repelleted. After one more washing, the digitonides were dissolved in 2 ml pyridine to regenerate sterols, and the regenerated sterols were silylated by addition of 100 μl BSA [N,*O-bis* (trimethylsilyl) acetamide] at room temperature as recommended by Grunwald (4). The silylated derivatives of sterol mixtures were chromatographed immediately on a 3% SE 30 column (column length 1.8 m and outer diameter 6.3 mm) in a Perkin-Elmer gas chromatograph model 3920 equipped with a flame ionization detector (FID). During chromatography, column temperature was 260°, and detector and injector temperatures were 280°. Flow rate of He was 50 ml/min^{-1} at 50 psi pressure. Retention times of individual sterols relative to 5 α-cholestane were: cholesterol=2.06, campesterol=2.64, stigmasterol=2.81, and sitosterol=3.24. Peak area was calculated according to the method of Grunwald (4).

Recovery of sterols was determined by using 5α-cholestane as internal standard. Generally, 75-80% recovery was observed. Accordingly, corrections were made for values obtained by gc by multiplying with a factor 4/3. The amount of the internal standard was such that it constituted about 25% of the amount (by weight) of the plant sterols present.

VERIFICATION OF METHODS.—During the course of this work, some questions arose as to the accuracy of the methods. Therefore, several experiments were conducted. The optimal amount of digitonin that would precipitate the maximal amount of sterols was investigated. When 1 ml of 2 $g/liter^{-1}$ or more of digitonin solution was added to 2 ml lipid extract of 1 g fresh weight tissue, maximal sterol recovery (70 μg) was obtained. When 100 μg of exogenous cholesterol was added in this sample at the first step of lipid extraction as a check on digitonin precipitation, we recovered 98.7% of the sterols. The digitonin itself did not interfere in the colorimetric determination of sterols. Further, standard sitosterol was determined by

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the colorimetric method with and without digitonin precipitation. Complete precipitation of sitosterol by digitonin was achieved over a wide range of concentrations. These experiments established the accuracy and completeness of sterol precipitation by digitonin from lipid extract.

DETERMINATION OF TOTAL PROTEIN.—Finely chopped leaves were homogenized with 10 ml of 0.1 N NaOH in a mortar at room temperature. The resulting slurry was stirred for 2 h at room temperature and then centrifuged at 30,000 g for 30 min. Proteins from the supernatant were precipitated with ice-cold 10% trichloroacetic acid and determined by a modified Lowry's procedure using bovine serum albumin as the standard (5).

CHANGES IN STEROL DURING LEAF AGING.—*S. nigrum* plants were grown in a greenhouse with a day-length extended to 16 h by supplemental illumination. Leaf samples were collected from 15-week-old plants. Leaves at three stages of development—i.e., young, mature, and old leaves—were collected. Because *S. nigrum* is a determinate plant growing from a succession of axillary buds, determination of leaf age on the basis of plastochrone index was not possible. Therefore, an arbitrary criterion was used (6). Leaves of the youngest branch were not fully expanded and were considered as young leaves, those of the next lower branch were fully expanded and were considered as mature leaves, and those on the next lower branch in the succession were the old leaves. Fresh and dry weights of leaves were determined, and the leaves were analyzed for β-C-3 sterols and protein contents.

CHANGES IN STEROL DURING IN VITRO MORPHOGENESIS.—Explants from young leaves of 15-week-old *S. nigrum* plants were cultured at 25 ± 1° at 16-h photoperiod on a basal medium containing MS salts (7), vitamins of B_5 medium (8), 3% sucrose, and 0.8% agar supplemented with either 10 μM indole acetic acid (IAA) or 10 μM benzyladenine (BA). At least ten explants were cultured in each treatment, and the entire experiment was repeated twice. After 33 days in culture, the roots and callus that formed on IAA medium and the shoots and callus on the BA medium were dissected, separately weighed, and analyzed for β-C-3 sterols.

RESULTS

CHANGES IN STEROLS DURING LEAF AGING.—Distribution of sterols in *S. nigrum* leaves indicates that, on a dry weight basis, the amount of total sterols was maximal in the mature leaves (Table 1). Significant variations occurred in case of individual sterols during leaf aging. Cholesterol was maximal in young leaves and declined significantly with aging. Sitosterol remained the predominant sterol in leaves of all ages. Highest content and proportion of campesterol and stigmasterol were detected in the mature leaves. Among all sterols examined, only cholesterol declined at successive stages of leaf aging. The relative amounts of C-29 sterols, stigmasterol, and sitosterol are expressed as their ratios to indicate their possible interconversion during leaf aging. The ratio in the young leaves was 0.1, which increased to 0.16 in the mature leaves followed by a decline to 0.07 in the old leaves (Table 1). The increased ratio in the mature leaves was due to a relatively enhanced accumulation of stigmasterol as compared to sitosterol. The decreased ratio in the old leaves was due to a decrease in stigmasterol and an increase in sitosterol.

The ratio of dry weight to fresh weight of 5-mm-diameter leaf disks taken from each type of leaf and the total protein content per gram of dry weight of leaves also declined during leaf aging. These data have been given as supporting evidence of the criteria of leaf aging.

CHANGES IN STEROL DURING IN VITRO MORPHOGENESIS.—Generally, tissue cultures raised on BA medium produced a greater amount of total sterols than those grown on IAA medium (Table 2). Total sterols of regenerated structures (roots and shoots) and of calli were significantly different from each other as well as from the original young leaves used to initiate cultures. Callus cultures had a content of sterols about two or three times lower than did the young leaves. The in-vitro-formed roots had a total sterol content more or less similar to that of young leaves. The regenerated shoots had about 150% more total sterol than did the young leaves because of a threefold and fivefold increase in campesterol and stigmasterol levels, respectively. Among the regen-

TABLE 1. Changes in β-C-3 Sterols in the Leaves of *Solanum nigrum* during Aging[a]

Leaf type	Fresh weight of 5-mm leaf disk (mg) ± SD	Dry weight 5-mm leaf disk (mg) ± SD	Protein content (mg/g dry weight)	μg/g dry weight ±SD					
				Cholesterol	Campesterol	Stigmasterol	Sitosterol	Total	Stigmasterol/ Sitosterol
Young leaves . . .	5.3±0.6	1.1±0.3	214	168.9±0.7 (13.5)[b]	92.0±34.2 (7.3)	88.6±39.9 (7.0)	901.1±25.5 (72.0)	1251.2± 99 (100)	0.10
Mature leaves . . .	7.6±1.5	1.2±0.4	151	119.0±3.1 (8.7)	130.4± 5.0 (9.6)	151.9± 6.3 (11.2)	957.1±15.9 (70.6)	1356.7±143 (100)	0.16
Old leaves	6.1±0.9	0.8±0.3	92	78.9±8.0 (6.0)	72.2±11.0 (5.6)	80.1± 6.8 (6.2)	1055.0±56.9 (81.4)	1295.5± 80 (100)	0.07
LSD	—	—	—	7.8	32.5	36.8	153.6	57.4	

SD=standard deviation.

[a]The results were analyzed statistically by calculated least significant difference (LSD) according to Snedecor (22).

[b]Figures in parenthesis indicate proportion of total sterols.

TABLE 2. Sterol Content of Young Leaf Explants of *Solanum nigrum* and of Tissue Culture Derived from Them

Tissue	µg sterol/g dry weight					
	Cholesterol	Campesterol	Stigmasterol	Sitosterol	Total	Stigmasterol/ Sitosterol
Young leaf	168.9± 0.7 (13.5)[a]	92.0±34.2 (7.3)	88.6±39.9 (7.0)	901.1± 25.5 (72.0)	1251.2± 99.7 (100)	0.10
Tissue cultures of 10 µM IAA medium						
A. Roots	134.5± 5.1 (10.0)	329.8± 4.3 (24.6)	272.5±19.1 (20.4)	600.3±10.3 (44.9)	1336.5± 28.5 (100.0)	0.45
B. Callus	37.2±14.2 (9.1)	61.5± 2.2 (15.0)	26.3± 4.4 (6.4)	283.2± 80.1 (69.3)	408.4±102.1 (100.0)	0.09
Tissue cultures of 10 µM BA medium						
A. Shoots	163.7± 7.1 (8.5)	269.7±92.6 (14.0)	456.9±98.6 (23.8)	1027.9±147.9 (53.6)	1918.0±346.2	0.44
B. Callus	64.4±22.7 (10.8)	102.3±22.7 (16.7)	178.0±41.6 (29.1)	265.8±97.5 (43.5)	610.4± 10.4	0.67
LSD	12.9	42.3	48.3	81.7	156.2	

[a]Figures in parenthesis indicate percent of total sterols.

erated structures, campesterol was significantly high in the roots, whereas stigmasterol was high in the shoots.

The total sterol contents of shoots and of the roots from seedlings were compared with the shoots and roots formed in vitro, each at two growth regulator concentrations (Table 3). The seedling roots contained about an eightfold higher amount of sterol than that of in-vitro-derived roots on 1 μM IAA medium and about a fourfold higher amount than that on 10 μM IAA medium. These differences were highly significant (Table 3). The differences between the sterol content of seedling shoots and in-vitro-derived shoots were less marked as compared with that of roots. The shoots formed on 10μM BA medium showed a significant difference in sterol content to that of seedling shoots. However, the shoots arising on 1 μM BA medium did not show any significant difference from that of seedling shoots.

The ratio of stigmasterol to sitosterol for roots and shoots did not differ significantly but differed considerably for the calli formed on IAA and BA media. The ratio for the callus formed on BA medium was about 7.5 times higher than the one formed on IAA medium, the reason being that the callus produced a greater amount of stigmasterol on BA medium than on IAA medium.

TABLE 3. Comparison of Total Sterol Content of Shoots and Roots Derived from Seedlings and In Vitro Cultures[a]

Plant part	Total sterol μg/g fresh weight
Shoots	
seedling derived	334.6[b]
in vitro formed shoots on 1 μM BA	271.3
in vitro formed shoots on 10 μM BA	95.3[b]
Roots	
seedling derived	565.5[c]
in vitro formed roots on 1 μM IAA	65.9[c]
in vitro formed roots on 10 μM IAA	120.6[c]

[a]Paired comparisons were statistically evaluated using *t* analysis, as suggested by Snedecor (22). Figures containing identical superscripts differ significantly from each other.

[b]Significant at $p=0.1$.

[c]Significant at $p=0.01$.

DISCUSSION

Changes in sterol composition during developmental processes such as seed germination (9), hypocotyl elongation (10), potato tuber formation (11), and leaf maturation (1) have been studied in order to understand the physiological function of sterols. The primary role of sterols is a metabolic one as architectural components of membranes (1, 2, 12). The actively dividing tissues maintain a higher rate of sterol synthesis to meet the rapid rate of membrane biogenesis (1, 13). The maturing tissues have a higher requirement for stigmasterol than they do for sitosterol (1, 14).

We have observed that as the leaf matures, the total sterol content reaches a maximum, as observed by other workers (2). Campesterol and stigmasterol are principally responsible for this increase. Cholesterol, total protein content, and ratio of dry to fresh weights decline as the leaf ages. Actively dividing tissue, like the young leaf, might be synthesizing more cholesterol, inasmuch as it is suggested that cholesterol

plays an important role in the structure of the membrane (15). Cholesterol is also a precursor for many C-29 steroids (16). We have observed a gradual decrease in solasodine (a steroidal alkaloid) in the leaves of *S. nigrum* during aging (Bhatt, unpublished). The decrease in solasodine content thus could be due to a decreasing level of cholesterol with aging. Campesterol decreases from mature to old leaves, and these data agree with the findings for aging leaves of soybean and tobacco (1, 2).

Our results suggest that the in vitro culture significantly alters the sterol composition of initial leaf explants and are, therefore, in agreement with earlier findings (17, 18). With BA in the medium, stigmasterol and sitosterol are produced at a significantly higher level in the regenerated shoot, whereas with IAA in the medium, a high level of campesterol was found in roots (Table 2). Further, the quantitative sterol compositions of in-vitro-formed roots and shoots are significantly different from those of the roots and shoots of newly germinated seedlings (Table 3). These results suggest that the growth regulators, especially IAA used in vitro culture, have a direct influence on the sterol metabolism. Brain and Lockwood (17) reported that callus and suspension cultures of *Trigonella foenum-graecum* grown on MS medium produced higher content of free sterol under the influence of 1 mg/liter 2,4-D but lower content after addition of 0.5 mg/liter kinetin or 10% (v/v) coconut water. They also reported that in many cultures, sterol level was higher than in the seed. Hardman and Stevens (18) found that in callus cultures of *Trigonella foenum-graecum*, sitosterol was predominantly produced with NAA in the medium whereas stigmasterol was predominant under the influence of 2,4-D. Although the plant tissue and growth regulator used by these workers differed from those used in our investigation, values for total and individual sterols are more or less comparable. Contents of cholesterol and campesterol are higher in *S. nigrum* cultures, as reported in this paper, than in the callus cultures of *Trigonella*, as reported by Hardman and Stevens (18). Further, contents of total sterol, stigmasterol, and sitosterol of *S. nigrum* callus cultures are higher than those of *Trigonella* cultures raised on NAA media.

The control of growth regulators on steroid production in undifferentiated cultures is much documented (19, 20), but comparison of steroid production in differentiated tissues vs. undifferentiated tissues has not been done in much detail. Previously, we reported higher production of the steroidal alkaloid solasodine in the differentiated cultures than in the undifferentiated cultures of *S. nigrum* (21). We here report that production of free sterols is also much higher in the differentiated cultures than in the undifferentiated cultures. Among the differentiated cultures, shoot-producing cultures produced a higher amount of total sterol than did the root producing cultures.

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LITERATURE CITED

1. C. Grunwald, *Phil. Trans. Roy. Soc. London*, **B284**, 541 (1978).
2. C. Grunwald, *Ann. Rev. Plant Physiol.*, **26**, 209 (1975).
3. M. Kates, in: "Laboratory Techniques in Biochemistry and Molecular Biology." Ed. by T.S. Work, E. Work, New York: North Holland-American Elsevier, 1972, p. 269.
4. C. Grunwald, *Anal. Biochem.*, **34**, 16 (1970).
5. E.F. Hartree, *Anal. Biochem.*, **48**, 422 (1972).
6. P.N. Bhatt, D.P. Bhatt, and I. Sussex, *Z. Pflanzenphysiol.*, **95**, 355 (1979).
7. T. Murashige and F. Skoog, *Physiol. Plant.*, **15**, 473 (1962).
8. O.L. Gamborg, R.A. Miller, and K. Ojima, *Exp. Cell Res.*, **50**, 151 (1968).
9. P.B. Bush and C. Grunwald, *Plant Physiol.*, **59**, 69 (1972).
10. J.M.C. Geuns, *Phytochemistry*, **12**, 103 (1974).
11. M.A. Hartmann and P. Benveniste, *Phytochemistry*, **13**, 2667 (1974).

12. W.R. Nes, *Lipids*, **9**, 596 (1974).
13. P. Duperon, *Physiol. Veg.*, **9**, 373 (1971).
14. D.L. Davis, *Phytochemistry*, **11**, 489 (1972).
15. C. Grunwald, *Plant Physiol.*, **48**, 653 (1971).
16. E. Heftmann, *Lloydia*, **38**, 195 (1975).
17. K.R. Brain and G.B. Lockwood, *Phytochemistry*, **15**, 1651 (1976).
18. R. Hardman and R.G. Stevens, *Planta Med.*, **34**, 414 (1978).
19. S.J. Stohs and H. Rosenberg, *Lloydia*, **38**, 181 (1975).
20. J.G. Marshall and E.J. Staba, *Phytochemistry*, **15**, 53 (1976).
21. P.N. Bhatt and D.P. Bhatt, *Physiol. Plant.*, **57**, 159 (1983).
22. G.J. Snedecor, "Statistical Methods," Ames: Iowa University Press, 1956.

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