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Effect of Light on Sterol Changes in *Medicago sativa*

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ABSTRACT

Most vascular plants contain Δ^5-sterols as the predominant type; however, a few species such as *Medicago sativa*, have mainly Δ^7-sterols. The Δ^7-sterols of alfalfa are isomers of the common Δ^5-sterols and are generally assumed to be their immediate precursors. Light had a significant influence on the sterol status of *M. sativa*. High light intensity and a long day favored the accumulation of dihydrospinasterol; a short day and low light intensity, particularly darkness, favored spinasterol accumulation. These data for Δ^7-sterol plants agree with those reported for Δ^5-sterol plants; light favors the accumulation of the monounsaturated 29 carbon sterols and darkness favors the accumulation of the diunsaturated sterols. Proposed is a mechanism to explain the effect of light on the accumulation of Δ^7- and Δ^5-sterols.

Sterols play an important structural role in the stabilization of membranes of vascular plants (8), and it has been suggested that changes in sterols alter cell permeability (8, 9). The amount of sterol and the composition of the sterol mixture are modified as a plant goes through its various stages of development or is exposed to changing environmental conditions (6, 9). Light is an important environmental factor that influences the biochemistry of plants, including the biosynthesis of sterols (3). Light, however, does not affect the status of individual sterols to the same degree. For example, shading field-grown *Nicotiana tabacum* increased the foliar stigmasterol level and decreased the sitosterol level (10). Similarly, shortening day length decreased the sitosterol level in *Solanum andigena* (2) and *Glycine max* (9); in wild potato, the decrease in sitosterol eventually reversed, but in soybean the sterol profile could only be altered by changing day length (C Grunwald, unpublished data). In tobacco, light has a greater effect on the biosynthesis of sitosterol than on the formation of stigmasterol (3), even though both are major components in tobacco and biosynthetically they seem to arise from a common precursor (12). All of the studies thus far have used plants that have Δ^5-sterols (2, 3, 9, 10). A few plant species, however, have Δ^7-sterols; that is, the ring double bond is between carbons 7 and 8 instead of carbons 5 and 6 (see Fig. 3, 24α-ethylcholesta-7,24(28)-dien-3β-ol for numbering of carbon atoms). Because the Δ^7-sterols are isomers of the Δ^5-sterols, they are generally assumed to be intermediates during isomerization of the $\Delta^8 \rightarrow \Delta^5$ bond (13, 14).

In the present study we examined the effect of light on the foliar sterol status of a Δ^7-sterol plant. Alfalfa was used as the experimental plant because its Δ^7-sterols spinasterol, dihydrospinasterol, and 24-methylcholest-7-enol are the respective isomers of the three common Δ^5-sterols stigmasterol, sitosterol, and campesteol, respectively, and thus correlative interpretations might be drawn.

MATERIALS AND METHODS

Plant Material. Alfalfa plants (*Medicago sativa* L. var Vernal) were grown in the greenhouse where natural light was supplemented with 110 to 112 μmol·photon m^{-2} s^{-1} of radiation at plant level provided by 400 W metal arc lamps. The plants were grown in 15 cm pots containing equal parts of loam, peat moss, sand, and vermiculite for at least 3 months before they were used in experiments. Plants were supplied complete fertilizer once per week. Shoots were cut regularly to keep the plants in a juvenile state. For experiments, freshly cut plants were transferred to a growth chamber and acclimated for 14 or 21 d at 25°C under a 12-h day produced by coolwhite fluorescent and incandescent lamps at 230 to 250 μmol·photon m^{-2} s^{-1}. The ratio of fluorescent to incandescent light intensity was about 10. After acclimation, plants generally had shoots 10 to 12 cm in length. Unless otherwise stated, six pots were used for each treatment. Composite samples were taken from each set of treatment pots. Each experiment was repeated at least three times.

Sterol Extraction and Analysis. Harvested shoots were homogenized with acetone and extracted in a Soxhlet apparatus for 18 h. The extracts were filtered and the appropriate internal standards were added for quantification: cholesterol for free sterols, cholesteryl palmitate for steryl esters, and cholesteryl glucoside for steryl glycosides. The steryl glycosides were separated from the free sterols and steryl esters by partitioning the sample between aqueous methanol (1:4, v/v) and hexane. The methanol phase contained the steryl glycosides; the free sterols and steryl esters remained in the hexane phase. Separation of the free sterols and steryl esters was by differential precipitation with digitonin. The dried hexane residue was dissolved in boiling 95% ethanol. Added were 0.25 volume of hot 2% digitonin in 95% ethanol and enough hot water to make the mixture 80% aqueous. After cooling overnight, the sterol-digitonide precipitate was washed three times with 80% ethanol and twice with diethyl ether. To recover the steryl esters, the mother liquid and washings were pooled, dried, and saponified for 30 min with 5% NaOH in 95% methanol. The freed steryl esters were precipitated with digitonin. The sterol-digitonide precipitates were broken with hot pyridine, and the digitonin was reprecipitated with diethyl ether.

Separation of Δ^5- and Δ^7-sterols was by TLC using Silica Gel G. The plates were developed four times with diethyl ether-benzene (1:9, v/v). The Δ^7- (slower moving, R_F 0.50) and the Δ^5-sterol zones (R_F 0.58) were visualized under UV light after spraying the chromatograms with a 0.5% solution of berberine in 95% ethanol. Sterols were extracted with methanol and partitioned into hexane. A known amount of cholestane was added for GLC quantification using a 15 m, 0.523 mm i.d. fused silica megabore DB-1 column. The column temperature was 260°C, with the injector and detector temperatures at 300°C. Carrier gas was He at 5 mL min^{-1}. Cholestane was used to quantify individual sterol components, and the cholesterol internal standard was used to calculate sterol recovery. Mass spectra were obtained by

Table I. *Foliar Sterol Composition of Greenhouse-Grown Alfalfa*

Plants were grown for 21 d at a day length of 12 h. Leaf moisture content was 85.3%.

Sterol	Free		Ester		Glycoside	
	$\mu g\ g^{-1}$ *fresh wt*	%	$\mu g\ g^{-1}$ *fresh wt*	%	$\mu g\ g^{-1}$ *fresh wt*	%
Δ^7-Sterols	104.2		14.88		0.110	
Spinasterol	74.2	71.2	10.24	68.9	0.083	74.8
Dihydrospinasterol	23.0	22.1	3.52	23.6	0.015	14.0
Methylcholest-7-enol	7.0	6.7	1.12	7.5	0.012	11.2
Δ^5-Sterols	1.89		0.478		Not detected	
Stigmasterol	0.75	39.9	0.097	20.3		
Sitosterol	0.80	42.2	0.304	63.6		
Campesterol	0.34	17.9	0.077	16.1		

Table II. *Effect of Continuous Illumination on the Δ^7-Free Sterols in Alfalfa Shoots*

Greenhouse-grown plants were acclimated for 21 d to a 12-h day before they were exposed to continuous illumination at 242 μmol·photon m^{-1} s^{-1}. Data are averages of three experiments. Values within a column followed by different letters are significantly different at the 0.05 probability level by Duncan's new multiple range test.

Continuous Light	Sterol Content	Methylcholest-7-enol	Spinasterol	Dihydro-spinasterol	Spinasterol/ Dihydrospinasterol Ratio
d	$\mu g\ g^{-1}$ *fresh wt*		%		
0	106.8 a	7.2 a	73.5 a	19.3 a	3.94 a
1	113.4 a	8.2 a	66.8 b	24.5 b	2.80 b
2	105.0 a	7.6 a	64.8 c	27.5 c	2.38 c

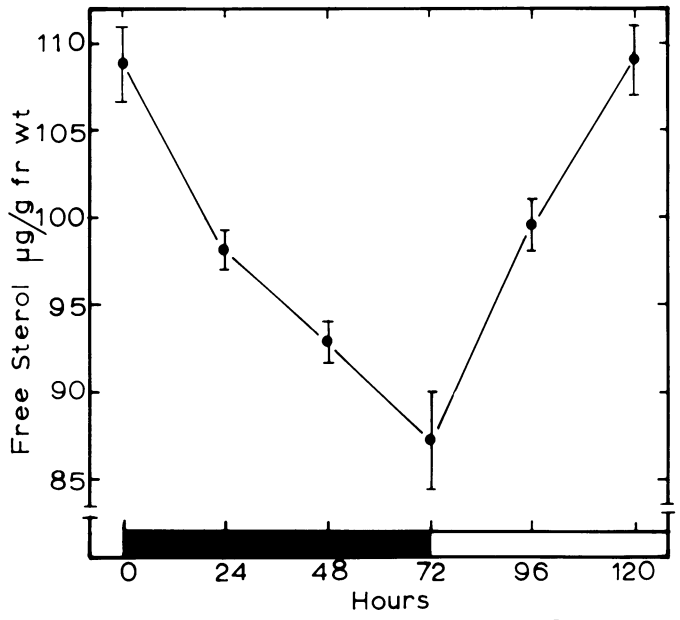

FIG. 1. Effect of light on the quantitative change of Δ^7-free sterols. Intact alfalfa plants were acclimated for 14 d at a 12-h day. Solid bar at the bottom of the figure shows plant exposure to darkness, open bar shows exposure to continuous illumination. Each point is the mean of three replicates, and verticals indicate standard error of the mean.

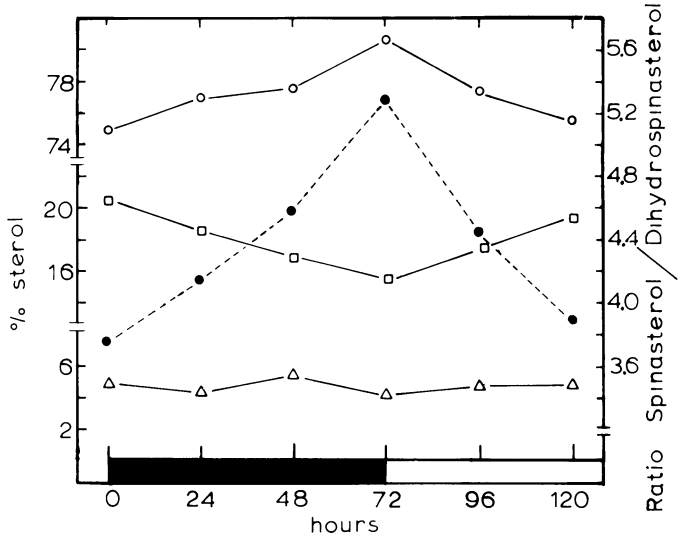

FIG. 2. Effect of light on the qualitative change of Δ^7-free sterols. Experimental conditions are the same as those described in Figure 1. Spinasterol (○), dihydrospinasterol (□), methylcholest-7-enol (△), and spinasterol to dihydrospinasterol ratio (●).

GC-MS using a 1.8 m, 4 mm i.d. glass column packed with 5% OV-101 and an ionization energy of 70 eV.

RESULTS

The shoots of greenhouse-grown alfalfa had both Δ^5- and Δ^7-sterols (Table I). The Δ^7-type accounted for 98% of total sterols, and the three major components, as confirmed by GC-MS, were spinasterol, dihydrospinasterol, and methylcholest-7-enol. Methylcholest-7-enol is similar to dihydrospinasterol except that it has a methyl at carbon 24 instead of the ethyl group. The Δ^5-sterols, which accounted for about 2%, were stigmasterol, sitosterol, and campesterol, and are the respective isomers of the Δ^7-sterols. Of the Δ^7-sterols, 87% occurred as free, with spinasterol the dominant component. The Δ^7-steryl esters accounted for 13%, and the composition of this fraction was quite similar to that of the free sterols. The Δ^7-steryl glycosides were a very minor component. Sitosterol was the major Δ^5-sterol component of the free and ester fractions, but the sterol compositions of the ester and free fractions were quite different. Glycosides of the Δ^5-sterols were not detected. In all further experiments only the Δ^7-free sterols were examined.

The shoots of alfalfa plants acclimated to a 12-h day for 21 d

Table III. *Effect of Day Length on Content and Composition of Δ^7-Free Sterols in Alfalfa Shoots*

Greenhouse-grown plants were acclimated for 14 d to a 12-h day before the day length was shortened to 8 h for 7 d, followed by a 4-h day for 7 d, and finally 7 d of darkness. Data are averages of three experiments. Values within a column followed by different letters are significantly different at the 0.05 probability level by Duncan's new multiple range test.

Day Length	Free Sterol Content	Methylcholest-7-enol	Spinasterol	Dihydrospinasterol	Spinasterol/ Dihydrospinasterol Ratio
h	*μg g^{-1} fresh wt*		%		
12	98.0 a	6.5 a	68.2 a	25.3 a	2.69 a
8	96.9 b	5.8 a	70.1 b	24.1 b	2.93 b
4	95.9 b	5.5 a	72.0 c	22.5 c	3.20 c
0	93.5 b	4.8 a	76.1 d	19.1 d	3.98 d

Table IV. *Effect of Light Intensity on Content and Composition of Δ^7-Free Sterols in Alfalfa Shoots*

Greenhouse-grown plants were acclimated for 21 d to a 12-h day. Light conditions were produced by fluorescent and incandescent lamps. The first reduction was in incandescent light and the second was in fluorescent light intensity. The final treatment was darkness. Plants were exposed to each condition for 3 d. Data are averages of three experiments. Values within a column followed by different letters are significantly different at the 0.05 probability level by Duncan's new multiple range test.

Light Condition	Light Intensity	Free Sterol Content	Methylcholes-7-enol	Spinasterol	Dihydrospinasterol	Spinasterol/ Dihydrospinasterol Ratio
	μmol·photon m^{-2} s^{-1}	*μg g^{-1} fresh wt*		%		
Incandescent and fluorescent	247	117 a	5.7 a	75.5 a	18.8 a	4.02 a
Incandescent and fluorescent	231	101 b	4.1 b	79.1 b	16.8 b	4.71 b
Fluorescent	227	112 ab	4.2 b	81.2 c	14.6 c	5.33 c
Fluorescent	194	105 b	4.6 ab	80.5 bc	14.9 c	5.28 c
No light	0	102 b	4.6 ab	82.6 d	12.8 d	6.43 d

showed a significant decrease in spinasterol and increase in dihydrospinasterol after exposure to continuous illumination, even though the total Δ^7-free sterols content had not changed (Table II). After 2 d in the light, the spinasterol level had decreased by 8.7% and the dihydrospinasterol level had increased by an almost equal amount. This change in sterol composition resulted in a decrease in ratio of spinasterol to dihydrospinasterol from 3.94 to 2.38. The methylcholest-7-enol level did not change during 2 d of continuous illumination.

When intact plants, acclimiated to a 12-h day for 14 d were transferred to darkness, the shoot showed a decrease in Δ^7-free sterols (Fig. 1). The decrease was almost 20% after 72 h. Returning the plants to light reversed the decline, and after 2 d of continuous illumination the foliar sterol content had returned to its original level. In the dark, spinasterol increased as percent of total free sterols, and dihydrospinasterol decreased. When illumination was resumed, the sterol composition returned to its initial level (Fig. 2). The percent change in sterols was small but significant, and is best observed when the data are expressed as a ratio of spinasterol to dihydrospinasterol (Fig. 2). During 72 h of darkness, the ratio increased from 3.75 to 5.24; when plants were returned to the light, the sterol ratio decreased to 3.89. The relative level of methylcholest-7-enol remained rather constant regardless of illumination.

To examine the effect of day length on sterol metabolism, shoots were removed from alfalfa plants, which were then grown under a 12-h day for 14 d. At this point a control shoot sample was harvested. The day length was then shortened to 8 h, and plants were kept under this condition for 7 d before the next harvest. After removal of a shoot sample, the day length was reduced to 4 h for an additional 7 d. As a final condition, plants were subjected to darkness for 7 d. The absolute sterol level decreased slightly with each reduction in day length; but of even greater importance, the relative level of spinasterol increased while that of dihydrospinasterol decreased (Table III). The spinasterol to dihydrospinasterol ratio under a 12-h photoperiod was 2.69, and it increased linearly ($R^2 = 0.908$) to 3.20 under a day length of 4 h. In darkness, the spinasterol to dihydrospinasterol ratio increased to 3.98. The relative and absolute level of methylcholesterol-7-enol was not affected by day length.

Light intensity also had an effect on the content and composition of Δ^7-free sterols (Table IV). Alfalfa plants grown for 21 d under a 12-h day with a light intensity of 247 μmol·photon m^{-2} s^{-1} produced by incandescent and fluorescent lamps had a spinasterol to dihydrospinasterol ratio of 4.02 (control). Reduction of the incandescent light intensity by 80%, to a total flux of 231 μmol·photon m^{-2} s^{-1}, increased the spinasterol level and decreased the dihydrospinasterol level. After 3 d, the spinasterol level had increased by 3.6% and the dihydrospinasterol level had decreased by 2.0%; thus the ratio increased from 4.02 to 4.71. Growing the plants for an additional 3 d under fluorescent lights only further increased the ratio of spinasterol to dihydrospinasterol to 5.33; however, lowering the fluorescent light intensity to 194 μmol·photon m^{-2} s^{-1} for an additional 3 d did not alter the ratio. The final condition, 3 d of darkness, resulted in an additional increase in spinasterol and a further decrease in dihydrospinasterol. It should be noted that the quantitative level of total Δ^7-free sterols level did not significantly change with reductions of light intensity.

DISCUSSION

It is well documented that in Δ^5-sterol plants light exerts a significant influence on the metabolism of sterols, particularly the two major 29 carbon components, sitosterol and stigmasterol (2, 3, 9, 10). A few plants, however, have only trace amounts of

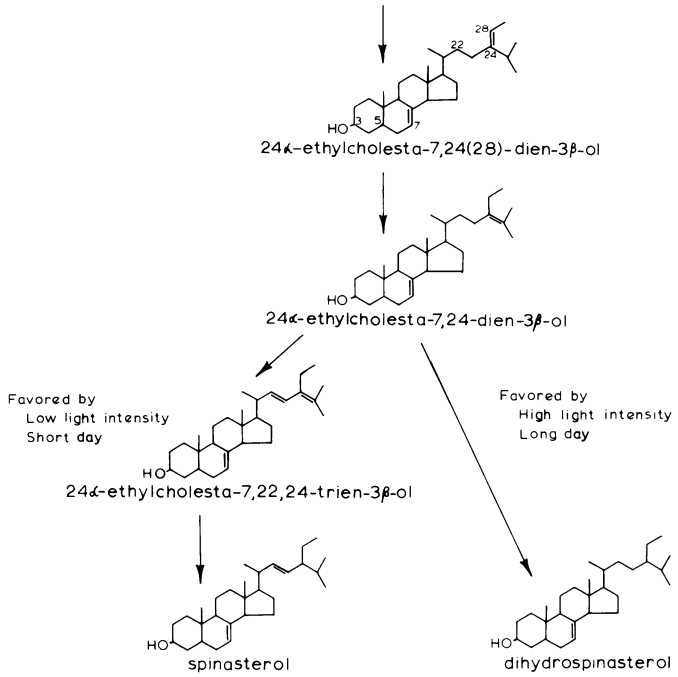

FIG. 3. Postulated light regulation of Δ^7-sterol biosynthesis in alfalfa. Numbered are key carbon atoms.

Δ^5-sterols. These plants have mainly Δ^7-sterols, and alfalfa is such a plant (Table I). The major 29 carbon Δ^7-sterols in alfalfa are spinasterol and dihydrospinasterol (1) and it has been postulated that they are in Δ^5-sterol plants the immediate biosynthetic precursors of stigmasterol and sitosterol. Apparently in Δ^7-sterol plants, such as alfalfa, the stereospecific isomerase fails to function properly (13, 14), thereby permitting the Δ^7-sterols to accumulate. We suggest that spinasterol and dihydrospinasterol in Δ^7-sterol plants perform the same function, that of controlling membrane permeability (8, 9), as do stigmasterol and sitosterol in Δ^5-sterol plants. This hypothesis is in compliance with the structural-functional relationships proposed for the Δ^5-sterols (8).

Data from the present investigation clearly show that the response of alfalfa to light is very similar to that of Δ^5-sterol plants. Light stimulated the foliar accumulation of the monounsaturated 29 carbon sterols, dihydrospinasterol in alfalfa (Table II), and sitosterol in Δ^5-sterol plants (3, 10), and darkness favored the accumulation of the diunsaturated sterols, spinasterol, and stigmasterol, respectively (Fig. 2). As with Δ^5-sterol plants, the change in foliar sterols induced by darkness was reversed when plants were again illuminated. Lengthening (Table II) or shortening (Table III) the day under which plants were grown also changed the spinasterol to dihydrospinasterol ratio. A longer day favored the accumulation of dihydrospinasterol, while a shorter day resulted in an increase in spinasterol. Similarly, light intensity had an effect on the sterol profile, that is, the higher the light intensity, the higher the foliar dihydrospinasterol level (Table IV).

The simplest explanation for the accumulation by alfalfa of dihydrospinasterol in the light and spinasterol in the dark is to postulate that light controls the interconversion of these two sterols. This hypothesis is appealing since both sterols have 29 carbon atoms and are identical in molecular structure, except that spinasterol has an additional double bond at C-22,23. Based on what is known about Δ^5-sterol plants; however, the interconversion of 29 carbon sterols is unlikely because the rate of incorporation of mevalonic acid into sitosterol and stigmasterol under conditions of light and darkness (3) does not support this hypothesis (9). Further, the direct conversion of sitosterol to stigmasterol could not be demonstrated in Δ^5-sterol plants (11, 13) and thus it has been suggested that in Δ^5-sterol plants, light affects the biosynthesis of sitosterol and stigmasterol independently (9). Furthermore, several investigators, based on circumstantial evidence, have suggested that in vascular plants the introduction of the double bond at C-22,23 occurs before the sterol side chain has been saturated (5, 7, 11, 12). This phenomenon would be analogous to the biosynthesis of ergosterol by yeast, where desaturation at C-22,23 occurs before C-24 saturation (4). In vascular plants, 24-ethylcholesta-7,24-dien-3β-ol has been reported to be an intermediate in the biosynthesis of the common 29 carbon Δ^5-sterols (15). We suggest that light controls whether the Δ^{24}-intermediate is saturated at C-24 to produce dihydrospinasterol or desaturated at C-22,23 to produce 24-ethylcholesta-7,22,24-trien-3β-ol (Fig. 3). Under the proposed hypothesis, a long day or high light intensity would favor saturation at C-24 and the formation of dihydrospinasterol; a short day or low light intensity would favor desaturation at C-22,23 and the formation of spinasterol. This hypothesis is attractive from an evolutionary standpoint because it would also explain the light effect in Δ^5-sterol plants since spinasterol and dehydrospinasterol are the precursors for stigmasterol and sitosterol, respectively.

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