Phytochemistry, Vol. 28, No. 2, pp. 465–468, 1989.
Printed in Great Britain.

0031-9422/89 $3.00+0.00

MEVALONATE INCORPORATION INTO ALFALFA STEROLS

LI-SHAR HUANG and CLAUS GRUNWALD

Illinois Natural History Survey and Department of Plant Biology, University of Illinois, Champaign, IL 61820, U.S.A.

(*Received* 16 *May* 1988)

Key Word Index—*Medicago sativa*; Leguminosae; alfalfa; light; mevalonate; Δ^7-sterols; spinasterol; 22-dihydrospinasterol.

Abstract—Light stimulated the uptake of mevalonate and its incorporation into the Δ^7-sterols of alfalfa. The increased incorporation of mevalonate into sterols in the presence of light was apparently due to a stimulation in 4,4-dimethyl sterol demethylation. Although spinasterol was the major sterol of alfalfa, 22-dihydrospinasterol had the higher specific radioactivity. Light, however, had a greater effect on spinasterol biosynthesis.

INTRODUCTION

The 4-demethyl sterols (sterols) of most vascular plants are of the Δ^5-type, and the most common are stigmasterol, sitosterol, and campesterol. A few species, however, have low levels of Δ^5-sterols; these plants have mainly Δ^7-sterols, and alfalfa (*Medicago sativa*) is such a plant [1]. The major Δ^7-sterols in alfalfa are spinasterol, 22-dihydrospinasterol, and 24-methylcholest-7-enol. The Δ^7-sterols are considered to be biosynthetic intermediates during the formation of Δ^5-sterols [2]. The influence of light on terpenoid metabolism in Δ^5-sterol plants has been well documented [3–8]. More recently we reported that the response of alfalfa to light was very similar to that of plants which produce Δ^5-sterols [9, 10]. We found that light stimulated the foliar accumulation of 22-dihydrospinasterol in alfalfa, and that darkness favoured the accumulation of spinasterol. Similarly, longer photoperiods favoured the accumulation of the former while shorter photoperiods resulted in an increase in the latter. Light had no effect on the foliar accumulation of 24-methylcholest-7-enol.

The effect of light on the incorporation of [2-^{14}C] mevalonic acid (MVA) by Δ^5-sterol plants has been examined [5, 6], but its effect on incorporation of MVA by Δ^7-sterol plants has never been studied. The objective of this communication is to report the effect of light on the incorporation of MVA by alfalfa and to draw comparisons between Δ^5- and Δ^7-sterol plants.

RESULTS AND DISCUSSION

Excised alfalfa leaflets floating on distilled water containing 0.1% Tween-20 and 0.6 μCi/ml [2-^{14}C]-MVA rapidly accumulated radioactivity. After 8 hr, leaflets incubated in light (Fig. 1A) had more than twice as much radioactivity as those incubated in darkness. The rate of ^{14}C incorporation into neutral lipids reflected the higher uptake of MVA in the light (Fig. 1A). However, its incorporation into unsaponifiable sterols was only 60% higher in light than in darkness (Fig. 1B). The higher uptake of MVA and its higher incorporation into neutral lipids had previously been reported with tobacco (*Nicotiana tabacum*), a Δ^5-sterol plant [6]. The foliar unsaponifiable sterol content of alfalfa was 115–121 μg/g fr. wt, of which 86–88% were free sterols (FS) and 12–14% were steryl esters (SE). During the first four hr of incubation, FS had a relatively lower rate of MVA incorporation than SE, but with longer periods FS showed a higher incorporation rate (Fig. 2A). The opposite observation was made with tobacco seedlings incubated continuously with MVA [6]. In alfalfa, light stimulated the incorporation of MVA into both FS and SE (Fig. 2A). A similar observation was made with tobacco [6]. However, in pot marigold (*Calendula officinalis*), another Δ^5-sterol plant, light had no effect on incorporation of MVA when the substrate was administered through the cut shoot [5]. As SE accounted for only 12–14% of unsaponifiable alfalfa sterols, they were not investigated further.

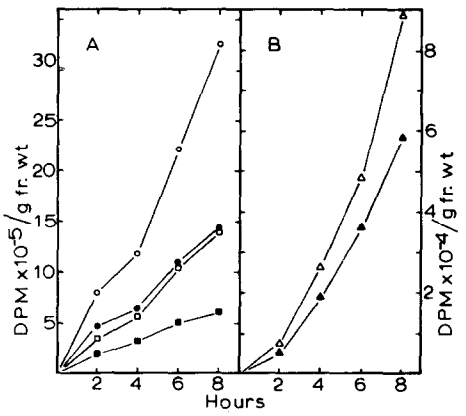

Fig. 1. Uptake and incorporation of [2-^{14}C]-MVA by alfalfa leaflets incubated in light (open symbols) or darkness (closed symbols). (A) Uptake: (○), (●); incorporation into neutral lipids: (□), (■). (B) Incorporation into unsaponifiable sterols: (△), (▲).

Ninety-eight per cent of the FS were Δ^7-sterols (69–72% spinasterol, 23–26% 22-dihydrospinasterol and 6–7% 24-methylcholest-7-enol) and 2% were Δ^5-sterols

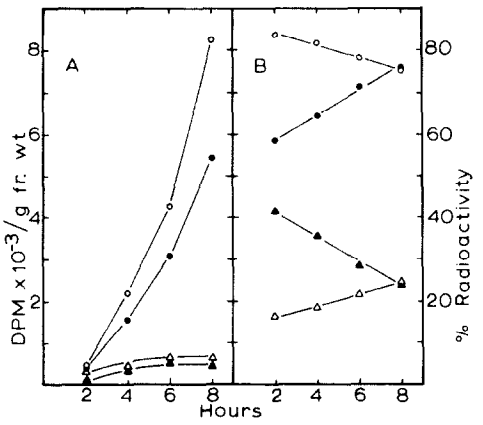

Fig. 2. Incorporation of [2-^{14}C]-MVA by alfalfa leaflets incubated in light (open symbols) or darkness (closed symbols). (A) FS: (○), (●); SE: (△), (▲). (B) % radioactivity incorporated into spinasterol: (△), (▲); into 22-dihydrospinasterol fraction which also contained 24-methylcholest-7-enol: (○), (●).

(46% sitosterol, 36% stigmasterol and 18% campesterol). After eight hr of continuous incubation with MVA, the Δ^5-sterols contained only trace amounts (<1%) of radioactivity; more than 99% of the ^{14}C activity was recovered from the Δ^7-sterols. Although spinasterol was the major Δ^7-sterol, its specific radioactivity was significantly lower than that of 22-dihydrospinasterol (Table 1). Since TLC was used to separate the mono- and di-unsaturated Δ^7-sterols, the 22-dihydrospinasterol fraction also contained the 24-methylcholest-7-enol. With incubation time, the specific radioactivity of both the spinasterol and the 22-dihydrospinasterol fractions increased, and light stimulated the incorporation of MVA, particularly its incorporation into the latter fraction (Table 1). A similar light-stimulated incorporation of MVA into the mono-unsaturated C_{29}-sterol (sitosterol) was reported for tobacco [6] and, in general, is in agreement with the light-induced foliar sterol accumulation pattern [7, 10]. The incorporation of MVA into the C_{29}-diunsaturated sterols under conditions of light and darkness is different for Δ^7- and Δ^5-sterol plants. In alfalfa, light stimulated the incorporation of MVA into spinasterol (Table 1), but in tobacco it did not affect its incorporation into stigmasterol [6].

Table 1. Specific radioactivity of the free sterols isolated from alfalfa leaflets incubated continuously with [2-^{14}C]-MVA in light or darkness

Incubation time (hr)	Spinasterol		22-Dihydrospinasterol*	
	Darkness	Light	Darkness	Light
	(DPM/μg sterol)			
2	12	9	93	133
4	45	62	377	657
6	109	114	825	1212
8	165	264	1583	2378

*22-Dihydrospinasterol fraction contained 24-methylcholest-7-enol.

The relative rate of biosynthesis of the various sterols in alfalfa was different under conditions of light and darkness (Fig. 2B). After two hr of darkness, 42% of the sterol ^{14}C activity was recovered from spinasterol and 58% from 22-dihydrospinasterol, but after eight hr of incubation, 24% of the radioactivity was found in spinasterol and 76% in 22-dihydrospinasterol. The reverse pattern of MVA incorporation was found in the presence of light. The per cent of ^{14}C recovered from 22-dihydrospinasterol decreased from 84% after two hr to 76% after eight hr, and the per cent of ^{14}C in spinasterol increased from 16% after two hr to 24% after eight hr. These radioactive labelling patterns are difficult to explain. A substrate to product relationship between 22-dihydrospinasterol and spinasterol is unlikely because the level of spinasterol has been shown to increase in darkness and that of 22-dihydrospinasterol to decrease. Thus, we might expect that in darkness the per cent radioactivity of spinasterol would increase with incubation; however, it did not (Fig. 2B). It has been suggested that the presence of MVA influences sterol biosynthesis [11], but the quantities of MVA used in our investigation would appear to be too low to have an impact.

Because light-stimulated uptake of MVA by alfalfa leaves made the interpretation of the biosynthetic results difficult, pulse-labelling studies were undertaken. A 30-min [2-^{14}C]-MVA pulse, administered in the light, resulted in the uptake of sufficient radioactivity to follow sterol biosynthesis for 24 hr (Table 2). As in continuous feeding studies, light significantly stimulated sterol biosynthesis and the stimulatory effect appeared to increase with incubation time. This observation led to the analysis of the 4,4-dimethyl, 4α-methyl, and 4-demethyl sterols (Table 3). Light had essentially no effect on total sterol biosynthesis when the radioactivity of the 4,4-dimethyl, 4α-methyl, and 4-demethyl sterols were considered together but clearly promoted demethylation of the 4,4-dimethyl sterols. The light-stimulated first demethylation became particularly evident after 3 hr of incubation. The second demethylation, that of 4α-methyl sterols, appeared to be favoured in darkness, but the overall effect was to inhibit the formation of 4-demethyl sterols in darkness.

As in continuous feeding experiments (Table 1), pulse-labelling studies revealed that MVA was incorporated into 22-dihydrospinasterol at a higher rate than into spinasterol, and light stimulated the biosynthesis of both sterols (Table 4). However, unlike the continuous labelling studies, the stimulatory effect of light was greater on spinasterol biosynthesis. The present data with alfalfa are difficult to reconcile in view of the fact that

Table 2. Specific radioactivity of the free sterols isolated from alfalfa leaflets incubated in light or darkness following a 30-min light-administered [2-^{14}C]-MVA pulse

Incubation time (hr)	Darkness	Light	Light stimulation (%)
	(DPM/μg sterol)		
1	109	131	20.2
3	222	297	33.8
5	397	545	37.2
24	601	834	38.8

Table 3. Incorporation of ^{14}C into 4,4-dimethyl, 4α-methyl, and 4-demethyl sterols by alfalfa leaflets incubated in light or darkness following a 30-min light-administered [2-^{14}C]-MVA pulse

Incubation time (hr)	Condition	Total sterol (DPM/g fr. wt)	Sterol form: 4,4-dimethyl %	4α-methyl %	4-demethyl %
0		103 851	84.1	9.2	6.7
1	Darkness	95 329	74.8	13.8	11.4
	Light	93 157	73.7	12.3	14.0
3	Darkness	79 713	66.8	17.7	15.5
	Light	83 145	40.8	23.5	35.7
5	Darkness	84 309	46.8	8.3	44.9
	Light	83 286	17.4	17.2	65.4
24	Darkness	81 344	26.9	6.2	66.9
	Light	84 186	13.5	2.5	84.0

Table 4. Incorporation of ^{14}C into free spinasterol and 22-dihydrospinasterol by alfalfa leaflets incubated in light or darkness following a 2-hr light-administered [2-^{14}C]-MVA pulse

Incubation time (hr)	Spinasterol Darkness (DPM/μg)	Spinasterol Light (DPM/μg)	Spinasterol Light stimulation (%)	Dihydrospinasterol* Darkness (DPM/μg)	Dihydrospinasterol* Light (DPM/μg)	Dihydrospinasterol* Light stimulation (%)
1	8	8	—	464	422	—
3	34	124	265	1377	1812	32
5	193	580	201	4750	5284	11
24	1476	2849	93	7376	8314	13
48	1230	2407	97	7087	7843	11

*22-Dihydrospinasterol fraction contained 24-methyl cholest-7-enol.

darkness stimulates the accumulation of spinasterol and light the accumulation of 22-dihydrospinasterol [10]. Researchers have suggested that spinasterol and dihydrospinasterol share a common Δ^{24}-sterol intermediate, possibly 24-ethylcholesta-7, 24-dien-3β-ol [12], and it may be that light favours saturation of C-24 to form 22-dihydrospinasterol. Darkness, on the other hand, promotes C-22/C-23 desaturation [10], resulting in the formation of spinasterol. Present data, however, do not support this hypothesis; in light and in darkness, incorporation of MVA was higher into 22-dihydrospinasterol than into spinasterol, and light promoted the incorporation of MVA into spinasterol. Taken in totality, the data cannot be explained unless separate metabolical sterol pools are postulated [13]. A similar approach was taken to explain the light effect on sterol metabolism in Δ^5-sterol plants [6].

EXPERIMENTAL

Plant material. Alfalfa plants (*Medicago sativa* L. var. Vernal) were grown in the greenhouse under a 12-hr photoperiod. Natural light conditions were supplemented by 400 W metal arc lamps with a flux of 250 μmol.photon/m^2/sec. To keep the plants in a juvenile stage and to delay flowering, the shoots were cut on a regular basis. For experimental purposes, leaflets from 14- to 21-day-old shoots were harvested.

Labelling studies. For continuous labelling studies, 0.5 to 0.6 g of alfalfa leaflets were incubated at 25° in 6 ml H_2O containing 0.1% Tween-20 and 10 μCi [2-^{14}C]-MVA (50.1 mCi/mmol). The light condition was 45 μmol.photon/m^2/sec. Samples were harvested at various times and washed ×4 with 250 ml H_2O, blotted, weighed, and homogenized twice in $CHCl_3$–MeOH (2:1) using manual glass grinders. In pulse-labelling studies, the 10 μCi [2-^{14}C]-MVA pulse was administered in the light for 30 min, whereupon the leaflets were extensively washed, transferred to 250 ml H_2O and incubated either in the light or in darkness. Harvested samples were washed and ground in $CHCl_3$–MeOH. To determine total MVA uptake, an aliquot was assayed for radioactivity.

Sterol separation. The $CHCl_3$–MeOH homogenate was filtered and washed twice with 0.2 vol. of 3 mM $CaCl_2$. The radioactivity of the organic phase was analysed to determine incorporation of MVA into neutral lipids. Separation of SE from FS was accomplished by differential ppt of sterols with digitonin [9]. Separation of 4,4-dimethyl, 4α-methyl, and 4-demethyl sterols (sterols) was by prep. TLC silica gel G, (0.5 mm), using $CHCl_3$–MeOH–HOAC (272:3:3) as eluent. Lanosterol and cholesterol were used as 4,4-dimethyl and 4-demethyl sterol markers, respectively. Separation of Δ^5- and Δ^7-sterols was by silica gel G prep. TLC (0.5 mm) developed in Et_2O–C_6H_6 (1:9). Spinasterol and cholesterol were used as respective markers. To separate the $\Delta^{7,22}$-from the Δ^7-sterols, the acetates were formed with Ac_2O–pyridine and separated by $AgNO_3$–silica gel G (1:8)

prep.TLC (0.5 mm) with dist. $CHCl_3$ (×4) as solvent. The acetates of stigmasterol and sitosterol were used as markers for the $\Delta^{7,22}$- and Δ^{7}-sterols, respectively. Sterols were visualized under UV light with a 0.05% soln of berberine in 95% EtOH. Sterols were extracted from the silica gel with MeOH and, after addition of H_2O to 20% aq., the sterols were partitioned into hexane, leaving the berberine in the MeOH phase. Scintillation spectrometry was used to determine radioactivity with correction for quenching. Sterol quantification was by GC, using cholestane as internal standard. The column was 15 m, 0.523 mm i. d. fused megabore DB-1. Column temperature was 260° with the injector and detector temp. at 300°. He at 5 ml/min was the carrier gas.

REFERENCES

1. Armarego, W. L. F., Goad, J. L. and Goodwin, T. W. (1973) *Phytochemistry* **12**, 2181.
2. Raab, K. H., deSouza, N. J. and Nes, W. R. (1968) *Biochem. Biophys. Acta* **152**, 742.
3. Bae, M. and Mercer, E. I. (1970) *Phytochemistry* **9**, 63.
4. Bush, P. B., Grunwald, C. and Davis, D. L. (1971) *Plant Physiol.* **47**, 745.
5. Kasprzyk, Z., Wojciechowski, Z. and Jerzmanowski, A. (1971) *Phytochemistry* **10**, 797.
6. Bush, P. B. and Grunwald, C. (1973) *Plant Physiol.* **51**, 110.
7. Grunwald, C. (1978) *Plant Physiol.* **61**, 76.
8. Izzo, R. and Navari-Izzo, F. (1981) *Plant Physiol.* **67**, 1073.
9. Huang, L.-S. and Grunwald, C. (1988) *Phytochemistry* **27** (in press).
10. Huang, L.-S. and Grunwald, C. (1988) *Plant Physiol.* (in press).
11. Hata, S., Shirata, K., Takagishi, H. and Kouchi, H. (1987) *Plant Cell Physiol.* **28**, 715.
12. Zakelj, M. and Goad, L. J. (1983) *Phytochemistry* **22**, 1931.
13. Goad, L. J. (1983) *Biochem. Soc. Trans.* **11**, 548.