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STEROL AND PHOSPHOLIPID CHANGES DURING ALFALFA SEED GERMINATION

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Key Word Index—*Medicago sativa*; Luguminosae; alfalfa; germination; phytosterols; Δ^7-sterols; phospholipids; spinasterol; dihydrospinasterol; phosphatidylcholine.

Abstract—Spinasterol, dihydrospinasterol, 24-methylcholest-7-enol, sitosterol, stigmasterol, and campesterol are in decreasing order the major sterols of *Medicago sativa* seeds. The first three are Δ^7-sterols and account for 94.4% of the total, while the latter three are Δ^5-sterols and represent 5.6%. During germination the total sterol level increased and the Δ^7- to Δ^5-sterol ratio remained constant. The steryl esters were the major sterol form and they increased during the first two days, while the free sterols increased at later stages of germination. During germination, spinasterol increased while dihydrospinasterol decreased. The major seed phospholipid was phosphatidylcholine, and its level increased during germination. Phosphatidylethanolamine and phosphatidylinositol occurred in about equal quantities; the former increased slightly while the latter decreased with germination. No correlation could be established during germination between various sterols and phospholipids and chlorophyll accumulation.

INTRODUCTION

Sterols are structural components of the lipid core of membranes and are involved in membrane stabilization [1, 2]. During plant development the sterol content is not static. Since membrane formation and transformation is an important early event of seed germination, a number of researchers have studied the biosynthesis and interconversion of sterols during this phase of development [3–8]. The sterols of vascular plants are a complex mixture, but in most species sitosterol, stigmasterol, and campesterol predominate. Since these sterols have a ring double bond between C-5 and C-6, they are referred to as Δ^5-sterols. A few plant species such as alfalfa (*Medicago sativa*), squash (*Cucurbita maxima*), and spinach (*Spinacea oleracea*) have very low levels of Δ^5-sterols (1–6% of total sterol). These plants have mainly Δ^7-sterols; the ring double bond is between C-7 and C-8 [9–12]. The Δ^7-sterols are considered to be biosynthetic intermediates during isomerization of $\Delta^8 \rightarrow \Delta^5$-sterols [13]. The two major Δ^7-sterols are dihydrospinasterol and spinasterol. Of the germination studies reported thus far, all but one [8] were carried out with Δ^5-sterol plants.

Garg and Nes [8] found that squash seeds had 78% Δ^7-sterol and 18% Δ^5-sterols, and that during the first nine days of germination the level of Δ^5-sterols decreased to less than 0.2% of total sterol even though the absolute sterol content per plant increased. Spinasterol was the major sterol and it increased during germination, but on a relative basis, dihydrospinasterol increased to a greater degree. During germination an increase in sterols has been reported for a number of Δ^5-sterol plants and, depending upon the species, either sitosterol or stigmasterol accounted for the major increase [5–7].

The present work was undertaken to expand the limited amount of information on changes in sterols during germination of Δ^7-sterol plants, and to correlate the sterol data with changes in phospholipids. Alfalfa was selected since, unlike squash, its main Δ^7-sterols are dihydrospinasterol, spinasterol, and methylcholest-7-enol which are the isomers of the common Δ^5-sterols, sitosterol, stigmasterol, and campesterol, respectively.

RESULTS AND DISCUSSION

Alfalfa seeds rapidly imbibed water and thus the ratio of dry weight to fresh weight decreased markedly during the first 24 hr of germination (Fig. 1). During the next

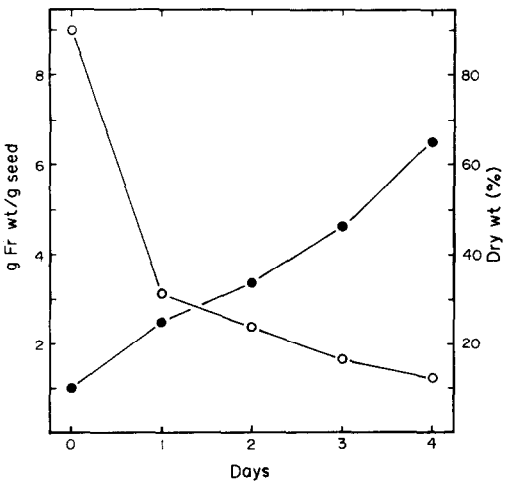

Fig. 1. Changes in fresh weight and percent dry weight during germination of alfalfa seeds. Data based on 1.00 g of initial seed mass: (●) fresh weight and (○) percent dry weight.

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three days of germination, the seeds accumulated water at a slower but linear rate until they reached a moisture level of 88–90%. Because of the rapid change in the ratio of dry to fresh weight, all data are presented on a per g of initial seed mass. There were about 460 seeds per g, and by visual inspection the seeds germinated uniformly with radicle protrusion occurring within 24 hr. The growth rate of the hypocotyls was linear between days one and four (Fig. 2).

The total chloroform–methanol extractable lipids accounted for *ca* 12% of seed mass, and the level did not significantly change during the first two to three days of germination. However, after four days the emerging seedlings had lost *ca* 20% of the initial seed lipids (Fig. 2). The seedlings started to accumulate chlorophyll on day three (Fig. 2), and reached their maximum level four days later. The phospholipids (PL) comprised *ca* 5% of extractable seed lipids and increased almost linearly during the four-day germination period (Fig. 3). The four-day-old alfalfa seedlings, on a per unit of initial seed mass, had almost twice as much PL as the seeds. Of the individual PL, phosphatylcholine (PC) accounted for 57%, and its relative level remained constant even though the absolute amount increased (Fig. 4). Phosphatidylinositol (PI) and phosphatidylethanolamine (PE) present at levels of 14 and 13%, respectively, were two other important PL in alfalfa seeds. During seed germinatin, the relative level of PI decreased slightly, while that of PE remained rather constant. Phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) were also identified; both occurred in the seed at relatively low levels but both increased during germination. The DPG level increased greatly during the first two days of germination.

The total sterol level of germinating seeds increased, over the four-day period, from *ca* 0.6 to *ca* 1.1% of extractable lipids (Fig. 3). The steryl esters (SE) accounted for almost half of the seed sterols and they, as a group, showed the largest increase during the first two days of germination. The free sterols (FS) were the second most abundant group. They did not increase significantly during the first two days of germination, but thereafter showed the largest increase. However, over the four-day germination period, the FS never reached the quantitative level of the SE (Fig. 3). The steryl glycoside (SG) level was *ca* 20% of the total seed sterols and remained at that relative level throughout the four days of germination.

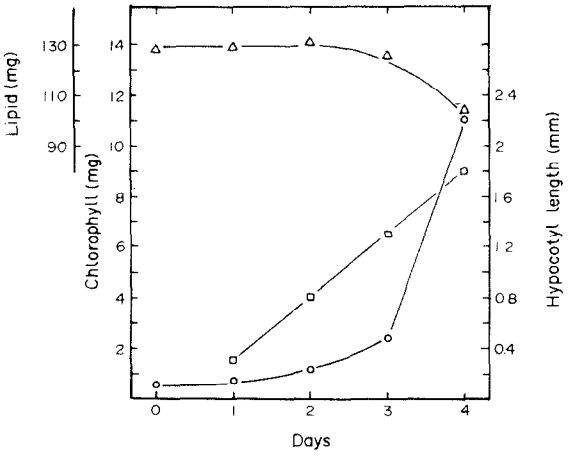

Fig. 2. Changes in pypocotyl length (□), total lipid content (△), and chlorophyll content (○) during germination of alfalfa seeds.

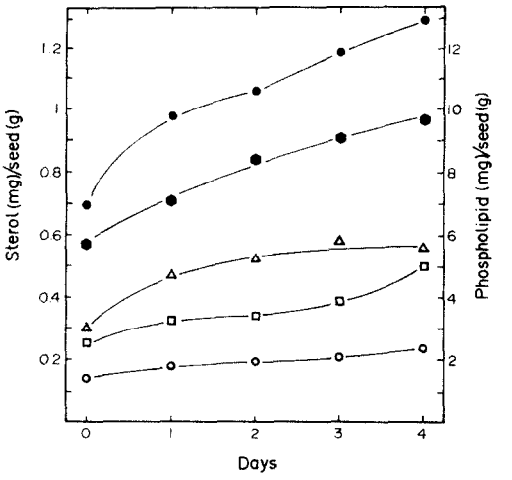

Fig. 3. Changes in the content of phospholipids (●), total sterols (●), SE (△), FS (□), and SG (○) during germination of alfalfa seeds.

The sterol increase during alfalfa germination closely paralleled the increase in total PL, especially after radicle protrusion (Fig. 3). The ratio of total PL to FS was constant during the first three days of germination and decreased thereafter; while the ratio of PL to SE decreased during the first day or two but remained constant thereafter. The change in ratio of PL to FS and SE reflects the shift in sterol accumulation from mainly SE during early germination to FS at later stages. Of the individual PL, PC and PE showed the same pattern as the total PL. However, the ratio of PI to FS or SE decreased with germination, and that of PG or DPG to FS or SE increased. Unfortunately, the present is the only available study, with either a Δ^5- or Δ^7-sterol plant, in which the various sterol forms and PL were monitored during early stages of germination and thus species comparisons cannot be drawn.

During germination the early increase of SE, and the later increase of FS, was also observed for two Δ^5-sterol species, *Nicotiana tabacum* [5] and *Sinapis alba* [7]. However, a number of other Δ^5-sterol plants did not have this sterol accumulation pattern [4, 14, 15]. The accumulation of FS in developing seedlings probably reflects the formation of membranes, since it is generally assumed that the free Δ^5-sterols play an important structural role in stabilizing plant membranes [1, 2]. We suggest that the Δ^7-sterols fulfill the same function in plants that are unable to synthesize sufficient quantities of Δ^5-sterol. This hypothesis is in compliance with the structural-functional relationships proposed for the sterols, in that ring B of the molecule must have at least one double bond [2].

The increase in SE during early stages of germination is difficult to explain. One suggested function for SE has been that they may play a role in the intra or intercellular transport of sterols [16]. In this scenario, during early phases of embryo axis growth, the sterol requirements are met by sterol transfer, in the form of esters, from area of storage to site of membrane formation [14]. If this

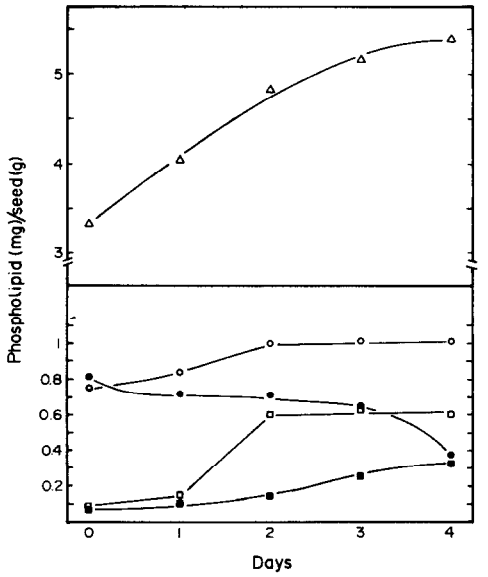

Fig. 4. Changes in the content of individual phospholipids during germination of alfalfa seeds. PC(△), PE (○), PL (●), DPG (□), and PG (■).

hypothesis is accepted, the FS should increase at the expense of SE; however, in the present investigation with alfalfa (Fig. 3) and in previous studies with tobacco [5] and white mustard [7], the FS increased but the SE did not decrease, and the individual sterols of the free and ester pools did not move in concert (Fig. 6). Another suggestion has been that the formation of SE may be a means of regulating sterol synthesis under conditions where excess sterols are produced [16, 17]. No direct evidence exists to support this hypothesis, but under this scenario the germinating seeds would accumulate sterols in the form of SE, which essentially have no effect on stabilizing membranes [1], and when needed the SE would be converted to FS. In the present investigation this demand for FS occurred as the seedlings started to green, on day 3 of germination (Fig. 2). During this period, free spinasterol doubled, yet the corresponding ester form did not decrease (Fig. 6). A similar lack of correlation was also found with tobacco [5].

Presently available data do not support a simple interconversion hypothesis of SE to FS. An interconversion argument for sterols can only be made if it is also assumed that dihydrospinasterol can be enzymatically converted to spinasterol. However, interconversion of the corresponding Δ^5-sterol isomer, sitosterol to stigmasterol, could not be demonstrated and it was suggested that introduction of the C-22 double bond occurred at a relatively early stage in the biosynthetic pathway [18].

The unsaponifiable sterol fraction of alfalfa seeds contained both Δ^7- and Δ^5-sterols. The former accounted for 94.5% and the latter for 5.5% (Table 1). This ratio of Δ^7- to Δ^5-sterol is in agreement with the published value for alfalfa seed oil [3]. During germination the quantities of both Δ^7- and Δ^5-sterols increased and after the first day the ratio of Δ^5- to Δ^7-sterol remained rather constant (Table 1). Capillary GC analysis of the Δ^7-sterol fraction revealed three major components and GC/MS gave fragmentation patterns corresponding to those reported for (in the order of GC elution) methylcholest-7-enol,

Table 1. Levels of unsaponifiable Δ^7- and Δ^5-sterols in germinating alfalfa seeds (data are the average of two experiments)

Germination period (days)	Total sterol	Δ^7-sterol	Δ^5-sterol	Δ^7-/Δ^5-sterol ratio
		(μg/g of seed)		
0	539	509	30	17.0
1	751	698	53	13.2
2	797	736	61	12.1
3	891	823	68	12.1
4	941	877	64	13.7

spinasterol, and dihydrospinasterol [12]. Armarego *et al.* [10] had previously identified these sterols in alfalfa. As previously found in the oil of alfalfa seeds, the Δ^5-sterols of the unsaponifiable seed lipid fraction were the common phytosterols, campesterol, sitosterol, and stigmasterol [11]. Figure 5 shows the relative change of the individual unsaponifiable Δ^7- and Δ^5-sterols with germination. Of the six sterols, spinasterol increased and dihydrospinasterol decreased, particularly after three days of germination, but the relative levels of the Δ^5-sterols remained constant.

Garg and Nes [8] had reported a rapid decrease in Δ^5-sterols during germination of squash, another seed that has mainly Δ^7-sterol. They observed that within five days of germination, the Δ^5-sterol level decreased from 18% to less than 1% of unsaponifiable sterols. In our study, even though the germinating alfalfa seeds synthesized sterols (Fig. 3), no decrease in the relative level of Δ^5-sterols occurred (Table 1). The Δ^7-sterols are considered to be biosynthetic intermediates during isomerization of $\Delta^8 \rightarrow \Delta^5$ [13]. Apparently in a few plant species, including alfalfa and squash, the stereospecific enzyme that removes the 5α- and 6α-hydrogens [19] is either inhibited

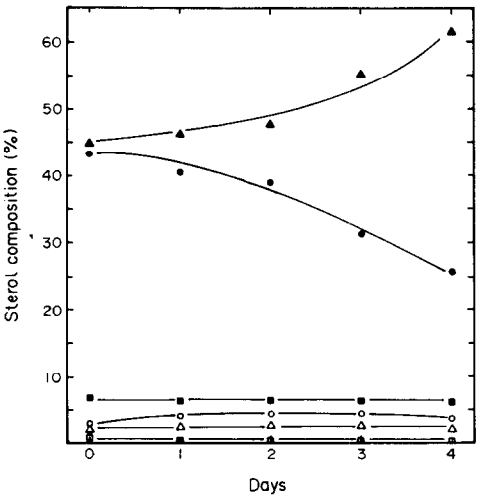

Fig. 5. Changes in the content of individual △^{7}- and △^{5}-unsaponifiable sterols during germination of alfalfa seeds. Spinasterol (▲), dihydrospinasterol (●), methylcholest-7-enol (■), stigmasterol (△), sitosterol (○), and campesterol (□).

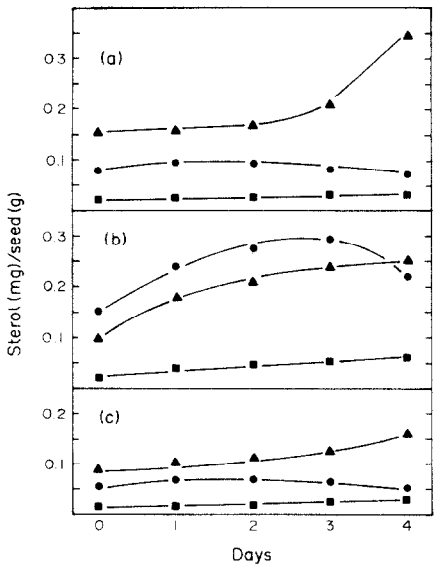

Fig. 6. Changes in the content of individual Δ^7-FS (a), Δ^7-SE (b), and Δ^7-SG (c) during germination of alfalfa seeds. Spinasterol (▲), dihydrospinasterol (●), and methylcholest-7-enol (■).

or operates at a very low level, and thus the Δ^7-sterols accumulate. Since alfalfa and squash seeds have Δ^5-sterols, the developing seeds must be able to synthesize these sterols to some degree. The synthesis of sterols during early germination, however, must be quite different for alfalfa and squash. In germinating squash the Δ^7- to Δ^5-sterol conversion is apparently inhibited and thus the ratio of Δ^5- to Δ^7-sterols decreased [8], but in germinating alfalfa the synthesis of Δ^5-sterols must have proceeded at a low rate and, therefore, the Δ^5- to Δ^7-sterol ratio did not change (Table 1, Fig. 5).

The quantitative changes during germination of the FS, SE, and SG are given in Fig. 6. During the first two days of germination no change in the amount of individual FS occurred (Fig. 6a), but starting with the third day, spinasterol increased from 154 to 351 μg/g of seed. During the same period, dihydrospinasterol decreased slightly and the level of methylcholest-7-enol remained constant. It was also during this period of germination that the seedlings started to accumulate chlorophyll (Fig. 2). However, the greening of the seedlings and the rapid increase in spinasterol is not directly related since the increase in spinasterol occurred whether the seeds were germinated in the light or in the dark, even though the total free Δ^7-sterol content was slightly lower in light-germinated seeds (Table 2).

At 56%, dihydrospinasterol was the major sterol component of the SE (Fig. 6b). Spinasterol accounted for 36% and methylcholest-7-enol for 8%. Quantitatively, all three SE increased during the first three days of germination but, on day four of germination, the dihydrospinasteryl esters started to decrease while the other two steryl esters continued to increase. The SG content was quite low (Fig. 6). As with the FS, spinasterol was the major sterol component of the glycosides (Fig. 6c) and it increased slightly with germination. Dihydrospinasterol was the second most dominant sterol of the SG and it decreased slightly with germination.

Table 2. Effect of light on the content of individual free Δ^7-sterols after four days of germination (quantitative data are averages of three experiments)

Incubation condition	Total sterol content (μg/g of seed)	Methyl-cholest-7-enol	Dihydrol-spinasterol (%)	Spina-sterol
Light	471 ± 14	7.1	13.6	79.3
Dark	511 ± 3	7.2	13.8	79.0

Garg and Nes [8], in their study with squash, did not separate the FS from the SE, but the unsaponifiable fraction revealed that during germination dihydrospinasterol increased at a faster rate than spinasterol. This is contrary to the observation with alfalfa (Fig. 4). Similar studies with Δ^5-sterol species have shown that either stigmasterol, as in tobacco [5], or sitosterol, as in wheat [14], can account for the major sterol increase during early stages of germination. These observations may, at first, appear to be inconsistent; however, it must be remembered that not all tissues of developing seedlings have the same sterol composition [3, 14], and analysis of whole plants represents an average. The compartmentalization and regulation of sterol synthesis in plants has been discussed by Goad [16], but any definite conclusions must await further experimentation.

EXPERIMENTAL

Plant material. Germination of alfalfa (*Medicago sativa* L. var. Vernal) was carried out by evenly distributing 1.00 g of seeds on wetted Whatman No 1. filter paper in Petri dishes. Incubation was at 25° with continuous illumination produced by cool-white fluorescent and incandescent lamps at 240 μmol-photon/m^2/sec. At various time intervals, the germinating seeds, including seed coats were harvested, weighed, and ground in the appropriate solvent using a polytron. For chlorophyll determination, the seedlings were extracted with 80% Me_2CO and the absorption determined at 645 and 663 nm [20].

Lipid extraction and analysis. The seedlings, derived from 1 g of seeds, were ground in $CHCl_3$–MeOH (2:1), filtered, and reground. The extract was washed twice with 0.2 vols of 3 mM $CaCl_2$. Total lipid determination was made gravimetrically by bringing the extract to constant weight at 80°. To determine total phospholipids, an aliquot of the lipid extract was dried and digested by gently refluxing until no further fumes were released, first with conc HNO_3 (60 min), followed by $HClO_4$. Phosphate content was determined spectrophotometrically at 820 nm using the molybdate procedure and KH_2PO_4 as the standard [21].

For individual phospholipid analysis, the crude lipid fraction, taken up in $CHCl_3$, was passed through a glass column (2 cm i.d.) packed with 2.6 g of silica gel (70–230 mesh) as a $CHCl_3$ slurry. The neutral lipids were eluted with 40 ml $CHCl_3$, and the polar lipids with 40 ml MeOH [22]. The MeOH fraction was dried, redissolved in a small amount of $CHCl_3$, and the phospholipids were sepd by 2D MgOAc–silica gel H (1:13) TLC [23]. The solvent in the first direction was $CHCl_3$–MeOH–28% aq. NH_3 (13:5:1), and in the second direction it was $CHCl_3$–Me_2CO–MeOH–HOAc–H_2O (6:8:2:2:1). The lipids were visualized with I_2 vapour and identified by co-chromatography with authentic standards. The desired spots were removed from the plate, digested, and assayed for phosphate.

Sterol extraction and analysis. The harvested seedlings were homogenized with Me_2CO and extracted in a Soxhlet apparatus for 18 hr. For quantification the appropriate int. standards were added: cholesterol for FS, cholesteryl palmitate for SE, and cholesteryl glucoside for SG. The dried extract was dissolved in hexane, and the SG were partitioned × 3 into 80% aq. MeOH. To free the sterols, the MeOH phase was brought to 0.5% H_2SO_4, refluxed for 12 hr, neutralized, extracted with hexane, and dried for sterol ppt. This procedure did not completely extract the SG, but by using the int. standard technique, proper corrections could be carried out. The FS and SE remained in the hexane. Unsaponifiable sterols were obtained by refluxing the dried hexane phase with 5% NaOH in MeOH for 30 min. If FS and SE separation was desired, fractionation of the hexane phase was by differential pptn with digitonin. The sterol residue was redissolved twice in boiling 95% EtOH, and pptd with digitonin in 80% aq. EtOH. After cooling overnight, the sterol–digitonide ppt. was washed × 3 with aq. 80% EtOH and × 2 with Et_2O. To recover the SE, the mother liquid and washes were pooled and the dried residue saponified with 5% NaOH in MeOH for 30 min.

Separation of Δ^5- and Δ^7-sterols was by silica gel G TLC. Development was × 4 with ET_2O–C_6H_6 (1:9). Sterols were visualized under UV light after spraying the chromatograms with a 0.05% soln of berberine in 95% EtOH. The Δ^7- and Δ^5-sterol (fast moving) zones were removed, extracted with MeOH, partitioned into hexane, and cholestane added as internal standard for GC quantification. GC analysis of the Δ^5- and Δ^7-sterols was by using a 15 m × 0.523 mm fused silica megabore DB-1 column. The column temperature was 260° with the injector and detector temperatures at 300°. Carrier gas was He at 5 ml/min. Mass spectra were obtained by GC/MS using a 1.8 m, 2mm i.d. glass column packed with 5% OV-101 and an ionization energy of 70 eV.

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