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Soybean sterol composition and utilization by *Phytophthora sojae*

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Abstract

The sterol fraction of *Glycine max* (soybean) was found to contain a mixture of 13 major sterols which differed dramatically in composition between seeds and shoots. Typical C4-desmethyl Δ^5-sterols, including sitosterol, predominate the sterol mixture of shoots, whereas C4-methyl sterol intermediates, cycloartenol and 24(28)-methylene cycloartanol, accumulate in seeds. The significance of modified sterol profile of shoot compared to seed was relevant to the physiology of *Phytophthora sojae*, a phytopathogen of soybean shown to be auxotrophic for sterol. Sterols native to the host plant containing a C4-methyl group, such as cycloartenol, were not utilized by the fungus. Alternatively, all Δ^5-sterols added to the culture media of *P. sojae* supported normal growth and promoted viable oospore production. The results demonstrate the importance of sterols in plant–fungal interactions and offer the possibility of bioengineering the phytosterol pathway for resistance to phytopathogens which scavenge specific sterols of the host plant to complete the life cycle.

Keywords: Soybean; *Glycine max*; Leguminosae; Host-pathogen interactions; Oomycetes; *Phytophthora sojae*; Sitosterol; Sterol

1. Introduction

The pythiaceous fungi (*Phytophthora* and *Pythium*) are a less-advanced group of phytopathogens which cannot synthesize sterols (Nes, 1987). This unusual defect in phytosterol synthesis by pythiaceous fungi has evolved into a nutritional dependency for Δ^5-sterols which can be acquired from the host during infection (Hendrix, 1970; Nes et al., 1982; Nes and Stafford, 1983). In the case of *Phytophthora sojae* which attacks soybean (*Glycine max*) (Erwin and Ribiero, 1996), Erwin et al. (1968) observed that the Δ^5-phytosterol, sitosterol can promote vegetative growth and sexual reproduction in an analogous manner to its effect on *P. cactorum* physiology (Nes et al., 1982). Based on these findings, we considered a hypothesis that Phytophthora-soybean interactions can be impaired by limiting sitosterol availability to the fungus. To test our proposal, we determined the phytosterol composition of soybean at two stages of development, one stage where no disease occurs — the seed, and a second stage where disease can cause serious crop losses — the shoot, and evaluated these materials as supplements in the growth media of *P. sojae*. Our results indicate that phytosterol intermediates which accumulate in seeds, C4-methyl sterols, can offer protection to disease and that limiting sitosterol availability can retard growth and interrupt oospore production.

2. Results and discussion

2.1. Sterol composition of soybean

The sterol composition of soybean seeds and shoots was determined by a combination of TLC, GLC, HPLC and MS as described in our earlier publications (Guo et al., 1995; Xu et al., 1988). In addition, the stereochemistry of the 24-alkyl group in the sterol side chain of material which had been purified by reversed-phase HPLC was established unambiguously by ^{1}H NMR (Guo et al., 1995). As shown in Table 1, the sterols of seeds and shoots are the same, but the proportion of the various sterols in the mixture and the total amount of sterol in each plant part differ significantly from seed to shoot (6-day seedling). To our knowledge, the elevated C4-methyl

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Table 1
Sterol composition of soybean shoots and seeds

Sterol	Structure	MW M^+	% Total sterol	
			Shoot	Seed
Cycloartenol	**1**	426	1	19
24(28)-Methylenecycloartenol	**2**	440	5	26
Obtusifoliol	**3**	426	1	tr.
14α,24-Dimethylcholest-8-enol	**4**	412	tr.	2
24(28)-Methylenelophenol	**5**	412	1	3
24(28)Z-Ethylidienelophenol	**6**	426	1	2
Isofucosterol	**7**	412	tr.	3
Sitosterol	**8**	414	61	23
Stigmasterol	**9**	412	17	3
Campesterol	**10**	400	5	9
24-Epicampesterol	**11**	400	5	9
Desmosterol	**12**	384	tr.	tr.
Cholesterol	**13**	386	tr.	0
Total sterol (μg/seedling)			23	60

sterol content of soybean seeds (at ca. 46% of total sterol) is the highest amount of sterol intermediates so far detected from crop plants. From our experience in analyzing phytosterol compositions of vascular plants, the amount of sterol intermediates in seeds is generally 10–20% of the total sterol mixture (Nes, 1990). The major sterols of soybean seeds were determined to be cycloartenol (**1**, Fig. 1) and 24(28)-methylene cycloartanol (**2**) whereas the major sterol of shoots was sitosterol (**9**). Fenner et al. (1986) determined that the shoot sterols of soybean remained in the same proportion to each other during the course of plant development.

2.2. Sterol biosynthetic capabilities of *P. sojae*

P. sojae was cultured initially in liquid culture on a maintenance medium to establish the native sterol composition of the mycelia. A non-saponifable lipid extract of the mycelia was analyzed by GC–MS and found to contain an identical sterol composition to that of the growth media viz., sitosterol, stigmasterol, campesterol and cholesterol (cf. Nes, 1987). Within the limits of detection of our chromatographic system no ergosterol was detected. These observations suggest the fungus lacks an active biosynthetic pathway to sterols. To confirm that *P. sojae* cannot synthesize sterol, the fungus was cultured in liquid medium with 10 ppm [2-^{3}H]lanosterol (9 Ci/Mol) derived from synthesis (Le and Nes, 1986) or [11–^{14}C] squalene (55 Ci/Mol, obtained from Research Products International Corp.). Both compounds were absorbed by the mycelia to ca. 5% of the fresh weight. The labeled material was recovered from the mycelia in unchanged form as indicated by a single band of radioactivity migrating on TLC plates corresponding to standards of lanosterol ($R_f = 0.5$) and squalene ($R_f = 0.9$), respectively (Xu et al., 1988). The radiotracer studies involving *P. sojae* are in agreement with our earlier observations with *P. cactorum* that Phytophthora cannot synthesize sterols de novo (Nes et al., 1986; Nes, 1987). In related studies, we (Nes et al. 1982; Stafford and Nes, 1983) and Gottlieb et al. (1978) found that *P. cactorum* and *P. infestans* can synthesize squalene but are unable to epoxidize the olefin to squalene oxide. Thus, the defect in the conversion of squalene to squalene oxide is likely the impaired step in the isoprenoid-sterol pathway responsible for sterol auxotrophy in this class of fungi.

To determine whether the fungus can metabolize sterols at all, the fungus was cultured on minimal broth with several soybean sterols, including cycloartenol, 24(28)-methylene cycloartanol, 24(28)-methylene lophenol, obtusifoliol, 24(28)-methylene cholesterol, sitosterol, and cholesterol (Fig. 1), and two sterols typically synthesized by fungi, lanosterol and ergosterol (Fig. 2). Each of the sterols was recovered from the fungus in unchanged form, except for ergosterol (M^+ 396 amu) which was metabolized to brassicasterol (M^+ 398 amu), the $\Delta^{5,22}$-analog of ergosterol, in 30% yield. The structure of brassicasterol was confirmed by its relative chromatographic mobility on TLC and HPLC and mass spectrum fragmentation pattern compared to that of an authentic specimen (Nes and Le, 1990). From these analyses, it would appear that *P. sojae*, like *P. cactorum* (Nes et al., 1982), has a limited number of enzymes than can act on the sterol substrate. In contrast to insects which are similarly auxotrophic for phytosterols (Nes et al., 1997) and convert sitosterol to cholesterol for hormonal purposes (Ikekawa et al., 1993), species of Phytophthora apparently utilize sitosterol in unchanged form to promote growth and sexual reproduction. These data and the fact sterols accumulate in mycelial membranes (Nes et al., 1982) indicate the primary role of sterols absorbed by Phytophthora is to affect membrane structure and function.

2.3. Sterol structure requirement of *P. sojae*

P. sojae was found to grow and reproduce sexually on a semisynthetic diet containing sitosterol as first reported by Erwin et al. (1968). We have now determined the amount of sitosterol required to promote maximal growth (as measured by hyphal extension) and oospore production is 10 mg/l or 0.001% of the diet (Figs. 3 and 4). In place of sitosterol a series of sterols were added to the fungal diet and their nutritional effects were evaluated. The sterols were chosen based on their relative position in the soybean sterol pathway as either a C4-methyl sterol intermediate or C4-desmethyl sterol end product. None of the C4-methyl sterol intermediates, cycloartenol **1**, 24(28)-methylene cycloartanol **2**, obtusifoliol **3** or 24(28)-methylene lophenol **5** promoted significant growth or induced functional oospore production. Sitosterol **8**, 24(28)-methylene cholesterol **14** and cholesterol **13** were absorbed by the mycelia to simi-

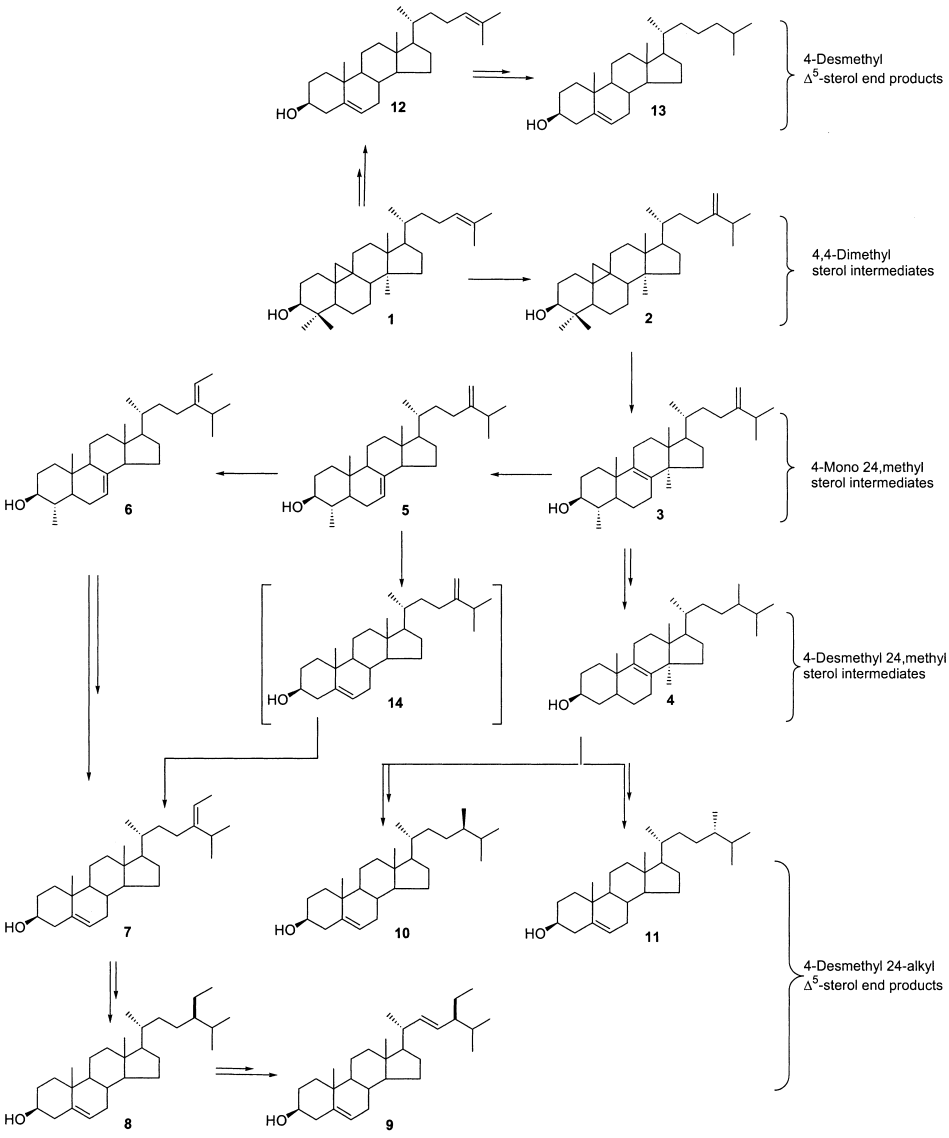

Fig. 1. Hypothetical sterol pathway in soybean. Compounds illustrated are: **1** cycloartenol, **2** 24(28)-methylene cycloartenol, **3** obtusifoliol, **4** 14, 24-dimethyl cholest-8-enol, **5** 24(28)-methylene lophenol, **6** 24Z-ethylidene lophenol, **7** isofucosterol, **8** sitosterol, **9** stigmasterol, **10** campesterol, **11**, 24-epicampsterol, **12** desmosterol, **13** cholesterol, **14** 24(28)-methylene cholesterol. Bracketed compound **14** represents a suspected intermediate in the pathway.

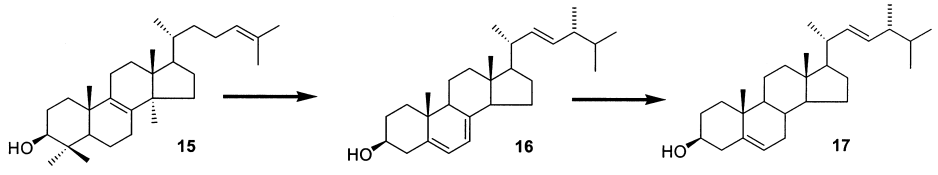

Fig. 2. Hypothetical pathway of fungal sterol biosynthesis. Compounds illustrated are: **15** lanosterol, **16** ergosterol and **17** brassicasterol.

lar levels, ca. 70 μg/g fr. wt, and cycloartenol and 24(28)-methylene lophenol were absorbed to a level of ca. 3 μg/g fr. wt. The amount of sterol absorbed by the mycelium was not necessarily limiting to phytosterol action, since cholesterol and 24(28)-methylene cholesterol were found to induce about half the number of oospores as were generated by treatment with sitosterol. These observations are similar to sterol-structure activity relationships observed with *P. cactorum* (Nes et al., 1982)

To establish the importance of sitosterol availability on *P. sojae* physiology the diet of *P. sojae* was supplemented first with different proportions of cycloartenol and sitosterol then with an equivalent amount of similar sterol types synthesized in the shoot and seed (Table 2).

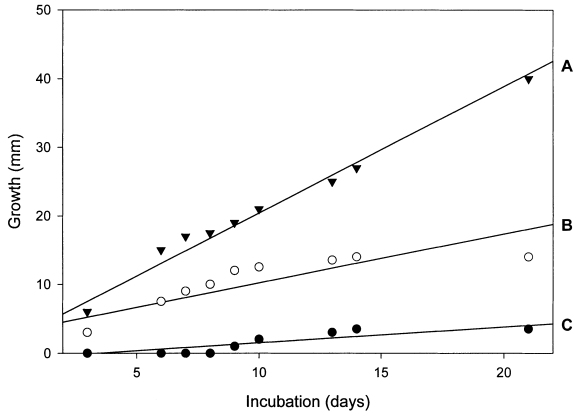

Fig. 3. Growth analysis (measured as hyphal extension) of *Phytophthora sojae* treated with 10 ppm sitosterol **A**, cycloartenol **B** or no sterol **C**. Petri plates were inoculated in the center of the plate with mycelia obtained from a maintenance culture using a 8 mm cork borer.

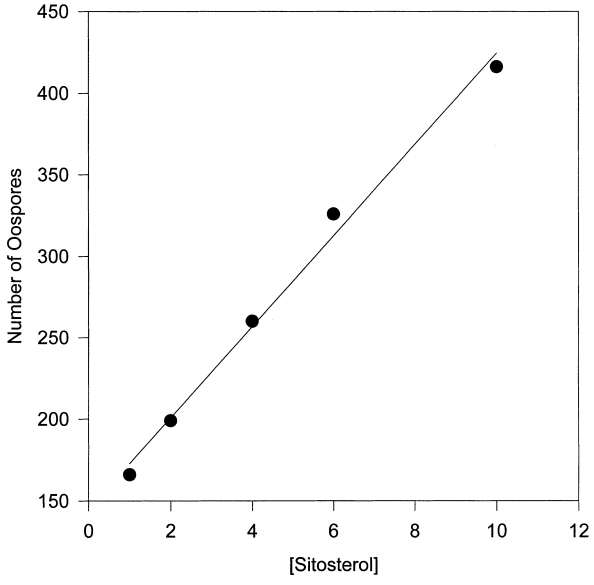

Fig. 4. Plot of increasing numbers of oospores per transect as a function of increasing amounts of sitosterol supplemented to the medium of *Phytophthora sojae*. The number of oopsores per transect remained constant at ca. 450 at higher levels of sitosterol treatment, ca. 15 or 25 ppm. Oospores were counted at 21 days of culture incubation. Microscope (Olympus Model CHT) settings for counting oospores were 40×.

Fungal growth was found to be significantly impaired at a 30/70 mixture of sitosterol to cycloartenol, which corresponds to ca. 50% the growth of sitosterol-treated mycelia. At a 10/90 mixture of the same set of sterols, growth of the mycelia was about the same as the 30/70 mixture of sitosterol to cycloartenol (data not shown). Growth of the fungus on a diet supplemented with 100% cycloartenol was very poor and not much better than the growth of a sterol-less diet, as reported in Fig. 3. *P. sojae* fed shoot or seed soybean sterol grew in a manner similar to mycelia fed a 50/50 mixture of sitosterol to cycloartenol. The number of oospores

Table 2
Phytophthora sojae utilization of cycloartenol spared with sitosterol

Sitosterol:cycloartenol ratio[a]	Oospores[b]	Sterol supplement[c]	
		Sitosterol	Cycloartenol
100:0	437	70	0
90:10	400	69	0
70:30	340	32	0
50:50	262	23	2
30:70	167	11	3
10:90	0[d]	5	3
0:100	0	0	3
NSF-shoot	423	58	0
NSF-seed	156	13	2

[a] Total sterol added to media at 10 ppm.
[b] Oospores per transect.
[c] μg/g fr. wt of mycelia.
[d] Media contained many aborted oogonia.

dropped from a maximum number of 437 to 0 as the proportion of sitosterol to cycloartenol was modified (Table 2). In the case of shoot sterol-treated mycelia where "sitosterol" levels are high in the sterol mixture, the number of oospores is high. However, in seed sterol-treated mycelia where "sitosterol" levels are low the number of oospores is also low. There is a clear correspondence between the amount of sitosterol in the sterol mixture, that amount of sitosterol absorbed by the mycelium and the number of oospores produced (Table 2). Whereas cycloartenol can be harmful to oospore production (Fig. 5), the amount of cycloartenol accumulated by the cells can be controlled as indicated by the limited absorption of C4-methyl sterols by the fungus.

One explanation for the low accumulation of cycloartenol by *P. sojae* is that species of Phytophthora synthesize a sterol carrier protein which can discriminate structural features of the sterol molecule. Indeed, Boissy et al. (1999) recently identified a pathogenic elicitin protein secreted by Phytophthora species which acts as a sterol carrier protein, favoring the binding over sitosterol to cholesterol and ergosterol. This sterol carrier protein is considered to facilitate movement of the host's sterol to the fungus. We hypothesize that a sterol carrier protein from *P. sojae* will be found to bind sitosterol with much greater efficacy than cycloartenol.

For these reasons, we assume that the unusual mixture of sterol in the seed fails to provide the necessary type or amount of phytosterol for *P. sojae* to grow and reproduce whereas the sterol mixture of the shoot can provide the nutritional sterol. Since the sterol methyl transferase (SMT) enzyme activity can regulate the cycloartenol-sitosterol pathway in soybean (Nes, 2000), it seems reasonable to hypothesize that bioengineering soybean to generate altered SMT levels could impair carbon flux thereby causing a change in phytosterol homeostasis to protect the plant from fungal attack.

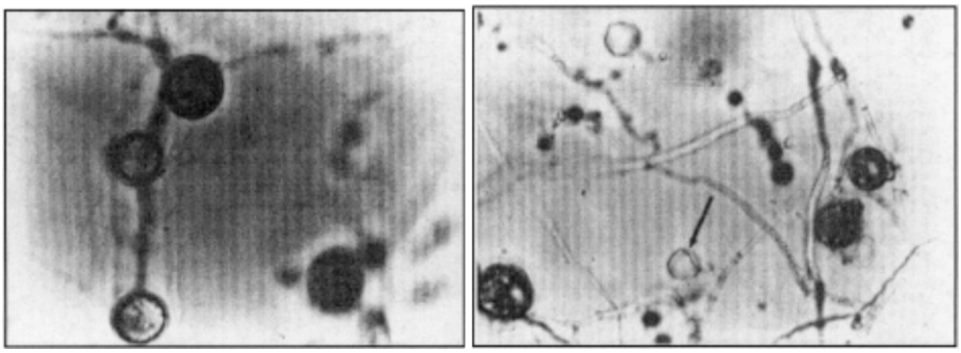

Fig. 5. Micrographs of normal oospores from (left picture) sitosterol-treated cultures and (right picture) aborted oospores from cycloartenol-treated cultures.

3. Experimental

3.1. General

The first set of experiments was designed to establish that *P. sojae* cannot synthesize sterols *de novo* and therefore, as in the case of other pythiaceous fungi, will require sterol to grow and reproduce sexually. To pursue these studies, *P. sojae* was cultured in two ways: Firstly, a maintenance system was developed which consisted of V8 juice/yeast potato dextrose medium added to agar distributed in 30 ml aliquots to 85 mm Petri plates. Secondly, a minimal broth was established which consisted of a synthetically compounded media to which was added sterol at 10 ppm (Nes et al., 1982).

P. sojae strain P7225 was obtained from Dr. Donald Erwin (Department of Plant Pathology at U.C. Riverside). The experimental methods for culturing *P. sojae* on solid and liquid substrates, determining growth rates, counting oospores and for isolating, identifying and quantifying sterols were as described in our earlier publications (Nes et al., 1982). Mycelia were grown in the dark at ambient temperature for 21 days. The sterols used in this study were from soybean or the Nes collection (Xu et al., 1988; Guo et al., 1995). Methods and instrumentation for mass spectroscopic and proton nuclear magnetic resonance spectroscopic analysis of sterols were as described (Guo et al., 1995). Samples for radiotracer analysis were dissolved in 5 ml of Scinti-Verse BD cocktail (Fisher). Radioactivity measurements were obtained on a Beckman LS 6500 liquid counter.

Soybean seeds strain DP3478 were purchased from Delta Pine and Land Co (Lubbock, Texas). Seeds were soaked in a 10% bleach solution for 10 min and washed. These seeds were imbibed with water and grown for 6 days in the dark. The resulting shoots were removed from the seeds and saponified in 10% methanolic KOH. The total sterol fraction was obtained from the non-saponifiable lipid fraction in the usual manner and purified into individual compounds by a combination of TLC and HPLC (Xu et al., 1988; Guo et al., 1995).

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