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Reassessment of the Role of Phospholipids in Sexual Reproduction by Sterol-Auxotrophic Fungi

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Several genera of oomycete fungi which are incapable of de novo sterol synthesis do not require these compounds for vegetative growth. The requirement for an exogenous source of sterols for sexual reproduction by several members of the Pythiaceae has been questioned by reports of apparent induction and maturation of oospores on defined media supplemented with phospholipids in the absence of sterols. A more detailed examination of this phenomenon suggested that trace levels of sterols in the inoculum of some pythiaceous fungi act synergistically with phospholipid medium supplements containing unsaturated fatty acid moieties to induce oosporogenesis. Phospholipid analysis of one species, *Pythium ultimum*, suggested that only the fatty acid portion of the exogenous phospholipid is taken up by the fungus. Enrichment of the phospholipid fraction of total cell lipid of *P. ultimum* with unsaturated fatty acids promoted oospore induction, and enhanced levels of unsaturated fatty acids in the neutral lipid fraction increased oospore viability. For some pythiaceous fungi, the levels of sterols required for the maturation of oospores with appropriate phospholipid medium supplementation suggest that these compounds are necessary only for the sparking and critical domain roles previously described in other fungi.

For over two decades it has been accepted that sterol-auxotrophic fungi of the Pythiaceae (Oomycetes) require an exogenous source of these compounds for sexual reproduction (14, 15, 19–21). Requirements for sterols have also been documented for asexual (11) and sexual (30) reproduction by the facultative mosquito parasite *Lagenidium giganteum* (Oomycetes:Lagenidiales), which cannot synthesize these compounds (60).

Several studies have questioned the absolute requirement of sterols for the production of viable oospores in several pythiaceous fungi (37–39). It has been claimed that the addition of phosphatidylcholine, phosphatidylethanolamine, and even some acylglycerols containing primarily unsaturated fatty acid moieties to defined culture media in the absence of sterols can induce oosporogenesis in several species of these plant-pathogenic fungi. A number of questions initially raised by Nes (47) concerning the presence of trace levels of sterols in even highly purified phospholipids (PL) led to a more detailed examination of this question.

In this study we examined the synergistic role of PL, fatty acids, and sterols in oosporogenesis by several sterol-auxotrophic fungi and confirmed the absolute requirement for the last class of compounds in this developmental process.

MATERIALS AND METHODS

Source and maintenance of fungal isolates. *Phytophthora cactorum* 1639, *Phytophthora megasperma* 1739, *Phytophthora citricola* 1745, and *Pythium ultimum* 1786 were obtained from E. Butler, Department of Plant Pathology, University of California, Davis. These plant-pathogenic fungi were maintained on 10% V8 juice agar (100 ml of V8 juice, 0.2 g of $CaCO_3$, and 20 g of Bacto-Agar [Difco Laboratories] per liter of deionized water) (38) or on PYG+ (1.25 g of Bacto-Peptone [Difco], 1.25 g of yeast extract, 1.25 g of glucose, 0.075 g of $CaCl_2 \cdot H_2O$, and 20 g of Bacto-Agar) supplemented with 10 mg of cholesterol, 2 ml of corn oil, and 1 ml of linseed oil per liter of deionized water (31). For evaluations of the roles of various lipids in oosporogenesis, the defined medium of Hohl (23) as modified by Ko (37) was used. It contained, in grams per liter of deionized water, the following: KNO_3, 0.1; KH_2PO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.05; $CaCO_3$, 0.1; asparagine, 0.1; glucose, 2; NaFeEDTA, 0.1; $CuSO_4 \cdot 4H_2O$, 0.1; $NaMoO_4 \cdot 2H_2O$, 0.005; $MnCl_2 \cdot 4H_2O$, 0.01; $Na_2B_4O_7 \cdot 10H_2O$, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.01; and thiamine hydrochloride, 0.1; it was adjusted to pH 8.0 with 0.5 N KOH prior to autoclaving. Agar media were solidified with either Noble agar (Difco) which had been washed two times for 24 h each time with 5% aqueous HCl in acetone or SeaKem agarose (37). Synthetic dioleoyl phosphatidylcholine and cholesterol, 99% pure, were obtained from Sigma Chemical Co. Cholesterol was further purified by recrystallization (two times) from absolute ethanol. PL were added to media prior to autoclaving by solubilization in a minimal volume of absolute diethyl ether (37). Cholesterol was added to media either in diethyl ether or after solubilization in 2 μl of tyloxapol-ethanol (1:1 [vol/vol]) per ml of growth medium (42).

Erlenmeyer flasks (250 ml), each containing 10 ml of defined medium were covered with aluminum foil and autoclaved for 15 min. Larger-scale incubations used foil-covered 2,500-ml Fernbach flasks each containing 200 ml of medium per flask. Media were inoculated with either 7-mm-diameter agar blocks dislodged from stock cultures by using a cork borer or the smallest quantity of hyphae possible obtained from liquid cultures by using a sharpened stainless steel spatula. Several of the cultures were initiated by inoculation of hyphal suspensions (3 ml/200 ml of culture or 0.3 ml/10 ml of culture) obtained by blending one 10-ml stock liquid culture in an additional 30 ml of sterile distilled water in a stainless steel blender. Liquid cultures used for inoculation were 10 to 14 days old, while agar inocula were from 4- to 8-day-old cultures. Cultures of the pythiaceous fungi were grown in darkness at 24°C without shaking.

Production and monitoring of oospores. Oosporogenesis was initiated for the pythiaceous fungi with a variety of inoculum sources and protocols. Initial evaluations with *P.*

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ultimum were done by following exactly the maintenance protocols described by Ko (37). The fungus was maintained for 10 days on V8 medium, and a 7-mm agar plug was transferred to defined agar medium (5 by 100 mm) and allowed to grow for 10 days. Agar cultures from four plates (20 ml of medium per plate) from which the inoculum plug and the central 1 cm had been removed were individually homogenized in a total volume of 50 ml of water in a Polytron tissue homogenizer (Brinkmann Instruments, Inc.), and oogonia, aborted oospores, and viable oospores were counted with a hemacytometer. Viability was based on easily recognizable morphological characteristics (30, 33, 46).

All other oosporogenesis evaluations were initiated by using one of the protocols described in the previous section and by following a variety of maintenance protocols to obtain sterol-free cultures. Cultures were constantly monitored following prolonged maintenance on sterol-free media to ensure that they retained the ability to enter the sexual cycle. This type of maintenance procedure can reduce or eliminate the ability of sterol-auxotrophic fungi to produce oospores under conditions usually conducive to this developmental option (30, 41, 50).

Production of oogonia and aborted and viable oospores was monitored 10 to 14 days after inoculation by diluting individual 10-ml cultures to a total volume of 40 ml and blending them at the highest speed in a Polytron tissue homogenizer. Quantitative estimates of these reproductive structures were made with a hemacytometer.

Lipid analyses. Sterols were extracted from mycelia by the acid labilization method of Gonzales and Parks (18). Mycelia were washed in 1% Tergitol-ethanol (1:1 [vol/vol]) and steamed for 20 to 30 min in 20 volumes of 0.1 N HCl. Mycelia were collected and suspended in 2 ml of 60% aqueous KOH–3 ml of methanol–2 ml of 0.5% pyrogallol. After refluxing with boiling stones for 2 h at 70 to 80°C, the saponification mixture was extracted three times with 10 ml of hexane. Occasional emulsions were cleared by the addition of a minimal volume of methanol.

Sterols were analyzed on a DB-5 or DB-17 capillary column (30 m by 0.25 mm [inner diameter]; J & W Scientific) with splitless injection and with H_2 as the carrier gas at 2 ml/min. A 5790A series gas chromatograph (Hewlett-Packard Co.) was operated at 140°C for 2 min, and the temperature was raised to 275°C at 15°C/min. The injection temperature was 250°C, and the flame ionization detector was operated at 300°C. Identification of sterols was based on relative retention times with cholestanol as an internal standard and initial analyses being done by gas chromatography (GC)-mass spectrometry (MS). GC-MS was performed with essentially the same operating conditions as those outlined above on a VG Masslab mass spectrometer operated at 70 eV and interfaced with a 5890A series gas chromatograph (Hewlett-Packard). Quantitation of sterols was done with an internal cholestanol standard or by direct computation with a Spectra Physics 4290 integrator programmed with a standard regression line generated from 25- to 200-ng cholesterol standards.

Total cell lipid was extracted by a slight modification of the method of Folch et al. (17). Cells were collected on a 100-μm mesh filter, washed with 1% Tergitol-ethanol–distilled water, and suspended in chloroform-methanol (2:1 [vol/vol]) with 0.01% butylated hydroxytoluene to minimize auto-oxidation. Cells were disrupted by blending in a Polytron tissue homogenizer and were filtered from the organic solvent. After partitioning against 2 ml of 0.75% aqueous KCl per 10 ml of chloroform-methanol, the lipid fraction was dried over anhydrous sodium sulfate and concentrated in vacuo at 35°C on a rotary evaporator. Lipids were taken up in a minimal volume of chloroform-methanol and either stored at −20°C or immediately processed further.

PL were separated from all other lipid classes by a modification of the solid-phase extraction method of Kaluzny et al. (27). Disposable aminopropyl columns (500 mg) with stainless steel frits were used with a Vac Elut vacuum elution apparatus and collection rack (Analytichem International). Initial extractions resulted in the elution from the columns of a mucilaginous nonlipid material in the methanol fraction which interfered with subsequent sample processing. This problem was minimized by an initial column wash with 3 ml of methanol and two subsequent washes under vacuum with 3-ml aliquots of hexane. Total lipid extracts in a minimal volume of chloroform-methanol were then applied to the column. The column was washed with two 3-ml aliquots of chloroform–2-propanol (2:1) and two 3-ml aliquots of 2% acetic acid in diethyl ether to elute all neutral lipids and unesterified fatty acids, respectively. PL were then eluted with two 3-ml aliquots of methanol. After drying over anhydrous sodium sulfate, the solvents containing the pooled neutral lipid-unesterified fatty acid fraction and the PL were dried under N_2 or with a rotary evaporator.

Lipid fractions to be analyzed for fatty acid content were derivatized by heating for 2 to 3 h at 100°C in 1 N methanolic HCl. The fatty acid methyl esters were extracted three times with hexanes, dried under a stream of N_2, and suspended in a minimal volume of hexane for GC or GC-MS. GC of fatty acid methyl esters was done with a DB-225 capillary column (30 m by 0.25 mm [inner diameter]) with H_2 as the carrier gas at 2 ml/min. The initial temperature of 140°C was held for 4 min and raised to 200°C at 4° C/min. Fatty acid methyl ester identification was based on relative retention times with heptadecanoic acid ($C_{17:0}$) as an internal standard. Chemical ionization GC-MS was used to confirm the identities of the fatty acids with operating conditions similar to those outlined above and the mass spectrometer described above. Isobutane was used for the chemical ionization.

PL classes were characterized with a variety of thin-layer chromatography systems (5, 56). Hard-layer silica gel HL plates (Analtech) were developed in solvent system 1 (acetone-chloroform-acetic acid-water [30:40:10:5]), solvent system 2 (chloroform–methanol–30% ammonium hydroxide [65:35:5]), or solvent system 3 (chloroform-methanol-acetic acid-water [63:38:9:3]). PL were visualized with iodine vapor. Dragendorff, ninhydrin, and periodate-Schiff reagents were used to confirm the identities of PL classes (57). Preparative thin-layer chromatography for quantification of PL used solvent system 1. Prescored plates were streaked with the PL fraction extracted from mycelia from 200 to 300 ml of liquid culture for each isolate. A single spot on the first quarter of each scored plate was exposed to iodine and used as a reference for subsequent scraping of the remainder of the plate.

Each plate was dampened with distilled water, the silica gel corresponding to individual PL classes was scraped into vials, and the lipids were extracted with chloroform-methanol. The filtered solvent was evaporated under N^2, and total phosphorous was estimated by the method of Mrsny et al. (45). Glassware was washed in 10% HCl in acetone–deionized distilled water. Water (50 μl) and perchloric acid (0.5 ml) were added to the PL fractions, vortexed, and digested in open-top tubes for 8 to 12 h at 130°C. After digestion 3.0 ml of water, 1.0 ml of 2.5% ammonium molybdate, and 0.5

ml of 10% ascorbic acid were added to the tubes, vortexed, and developed for a minimum of 1.5 h in a 37°C water bath. A standard curve was generated with $NaH_2PO_4 \cdot H_2O$. Phosphomolybdate color was measured at 820 nm with a Cary 14 UV/VIS spectrophotometer or a Spectronic 21 apparatus (Milton Roy).

Lipid standards and medium supplements were obtained from Sigma or Nuchek Prep, with the exception of ceramide aminoethylphosphonate, which was the generous gift of R. Bostock.

RESULTS

Initial screening of pythiaceous fungi. Twelve species of pythiaceous fungi maintained on V8 juice agar were screened for oospore formation on defined agar media supplemented with 1 g of dioleoyl phosphatidylcholine (lecithin) per liter following a single passage of stock cultures on sterol-free culture media. Four species which produced significant numbers of oospores under these conditions were chosen for further investigation.

When the Ko (37, 38) protocol for inoculating defined media was followed, sufficient lipid supplement was present in a single 7-mm agar inoculum plug from V8 juice medium to induce appreciable oosporogenesis on defined agar media lacking any exogenous lipid supplement. When a culture of *P. ultimum* maintained on a defined medium lacking lipid supplement for six generations was transferred to V8 juice medium and then back to a defined medium lacking lipid supplement, $(1.6 \pm 1.1) \times 10^4$ viable oospores, $(3.1 \pm 1.7) \times 10^4$ aborted oospores, and $(8.1 \pm 1.0) \times 10^4$ oogonia per agar plate (20 ml of medium, $n = 4$) were formed on plates, not including the 1-cm area adjacent to the site of inoculation. No viable oospores were formed on the same defined media inoculated with agar plugs from plates maintained for six generations in the absence of lipid supplementation.

These results imply that the protocol followed by Ko (37, 38) for the induction of oosporogenesis on defined media is complicated by the presence of compounds that are sequestered by mycelium and that are sufficient to induce sexual reproduction in the absence of any exogenous lipid or other medium supplements.

Sterols were present in lecithin-supplemented media which induced viable oospores. After a single passage from V8 juice medium to basal agar medium, 10 7-mm agar blocks of a *P. ultimum* culture from the defined agar medium were used to inoculate a 200-ml culture consisting of basal medium plus 1 g of dioleoyl phosphatidylcholine per liter. This was ca. half the amount of inoculum used by Ko (37) in his oospore evaluations. Mycelium from a 10-day-old culture of *P. ultimum* grown under these conditions contained 8.9 μg of total sterol. The sterol composition of the fungus approximated that found in V8 juice, i.e., relatively high amounts of sitosterol and stigmasterol and smaller quantities of campesterol and cholesterol (47). An ethereal extract of V8 juice used in these experiments revealed the following sterol composition: 50.5% sitosterol, 34.2% stigmasterol, 13.7% campesterol, and 1.6% cholesterol.

The four species of plant-pathogenic fungi were analyzed for their capability to sequester and transport sterols. After 30 passages on a defined liquid medium lacking lipid supplement, the fungi were transferred to V8 juice medium. A single 7-mm plug from 7-day-old cultures was placed in the middle of a 100-mm agar plate containing the defined medium of Ko (37). After 4 days of growth, a radial section of each culture starting 1 cm and extending to 2 cm from the edge of the V8 juice inoculum was collected from each of two agar plates, and the content of nonsaponifiable lipids was examined.

All species sequestered sterols and transported appreciable quantities a distance comparable to that used by Ko (37) for his "sterol-free" experiments. The total sterol found for each species was as follows: *P. ultimum*, 10,787 ng (53.5% sitosterol, 32.5% stigmasterol, 10% campesterol, and 4% cholesterol); *P. cactorum*, 2,443 ng (46.3% sitosterol, 43% stigmasterol, 10.7% campesterol, and a trace of cholesterol); *P. megasperma*, 1,078 ng (51.8% sitosterol, 36.7% stigmasterol, 11.5% campesterol, and a trace of cholesterol); and *P. citricola*, 898 ng (38.6% sitosterol, 32.3% stigmasterol, and 29.1% campesterol).

A second series of experiments examined the amounts of sterols present in defined agar media after transfer of a culture of *P. ultimum* from PYG+ to basal medium to demonstrate that sterol sequestration was not an artifact of the culture protocol. Extraction of concentric rings after 6 days of growth from a central agar inoculum plug revealed 1,031 ng (primarily cholesterol) in the central 1 cm surrounding the inoculum, 196 ng between 1 and 2 cm from the center, 48 ng between 2 and 3 cm from the center, and 53 ng in the outermost ring. Confirmation of the sterol structures was made by GC-MS in all instances. These experiments suggested that there were significant levels of sterols in all cultures of pythiaceous fungi when the protocol of Ko (37, 38) was used to test the effect of PL on oosporogenesis.

A detailed evaluation of the effect of the culture protocol on PL induction of oosporogenesis revealed that after a single passage from sterol-containing medium (PYG+) to either defined agar medium or liquid medium, dioleoyl phosphatidylcholine-supplemented media initiated oospore induction and maturation in the three *Phytophthora* species and the *Pythium* species (Table 1) in the absence of exogenous sterols. Sterols were present, however, in trace quantities in the agar inoculum and probably in the liquid culture inoculum, as detailed above. After seven passages on defined media in the absence of lipid supplementation, no sterols were detected in the inoculum, and only two species, *P. cactorum* and *P. ultimum*, produced viable oospores on dioleoyl phosphatidylcholine-supplemented media (Table 1). When this lot of dioleoyl phosphatidylcholine was further purified on aminopropyl columns, this vestigial level of oosporogenesis disappeared. It is assumed that trace levels of contaminating sterols were responsible for the production of oospores.

Highly purified soybean lecithin has been shown to contain detectable levels of sterols (47). Two separate lots of synthetic dioleoyl phosphatidylcholine (catalog no. P-1013, 99% pure; Sigma) contained 631 ng of cholesterol/g and 587 ng of cholesterol/g when analyzed by GC-MS. The source of the phosphocholine headgroup for this synthetic product is egg yolk (Sigma Chemical Co. Technical Service, personal communication), which explains the trace quantities of cholesterol present.

Cholesterol added at 10 mg/liter induced viable oospore production in all isolates (Table 1). The numbers of oospores induced by cholesterol were reduced after seven passages on defined medium without lipid supplement (Table 1, culture protocol C) but remained at significant levels.

In analyses of sexual reproduction it is essential to differentiate between the initiation of oogonia and oospores and their subsequent maturation. The former developmental events can be initiated in the absence of sterols or by fairly nonspecific polycyclic isopentenoids (13, 16, 50), but com-

TABLE 1. Effect of culture protocol on sexual reproduction by four species of sterol-auxotrophic fungi

Culture protocol	Species	Medium supplement[a]		No. of the following produced/ml[b]:		
		Cholesterol (mg/liter)	Lecithin (g/liter)	Oogonia	Aborted oospores[c]	Viable oospores
A[d]	*Phytophthora cactorum*			4.4×10^3	4.0×10^3	6.6×10^2
			1.0	3.5×10^4	4.8×10^3	4.0×10^3
		10.0		3.0×10^3	1.5×10^4	4.8×10^4
		0.1	1.0	7.0×10^3	1.7×10^4	2.3×10^4
	Phytophthora citricola			2.1×10^3	5.1×10^2	0
			1.0	8.8×10^3	5.2×10^3	3.5×10^3
		10.0		6.5×10^3	9.5×10^3	4.1×10^3
		0.1	1.0	1.4×10^4	2.9×10^4	5.0×10^3
	Phytophthora megasperma			7.6×10^2	7.6×10^2	0
			1.0	0	2.4×10^3	1.0×10^3
		10.0		1.3×10^3	5.2×10^3	3.2×10^4
		0.1	1.0	1.1×10^3	2.9×10^3	5.9×10^3
	Pythium ultimum			1.1×10^5	8.0×10^2	0
			1.0	1.9×10^5	1.0×10^5	1.8×10^5
		10.0		7.0×10^3	1.6×10^4	2.3×10^5
		0.1	1.0	1.9×10^5	6.0×10^4	3.5×10^5
B[e]	*Phytophthora cactorum*			0	0	0
			1.0	1.0×10^4	8.5×10^3	0
		10.0		2.9×10^3	1.1×10^4	2.2×10^4
		0.1	1.0	6.8×10^3	6.7×10^3	3.7×10^4
		10.0	1.0	1.2×10^4	3.4×10^4	1.2×10^5
	Phytophthora citricola			0	2.4×10^3	3.8×10^3
			1.0	5.9×10^3	1.2×10^4	1.1×10^4
		10.0		0	8.0×10^3	2.5×10^4
		0.1	1.0	8.2×10^3	1.8×10^4	2.9×10^4
		10.0	1.0	1.1×10^3	7.0×10^4	5.3×10^4
	Phytophthora megasperma			0	2.8×10^3	2.8×10^3
			1.0	0	6.8×10^3	3.2×10^3
		10.0		0	1.8×10^4	7.6×10^4
		0.1	1.0	0	5.0×10^3	4.9×10^4
		10.0	1.0	0	1.2×10^3	2.1×10^5
	Pythium ultimum			1.5×10^4	4.7×10^3	3.4×10^4
			1.0	1.3×10^5	2.0×10^4	2.9×10^4
		10.0		7.7×10^3	1.1×10^4	1.0×10^5
		0.1	1.0	0	1.2×10^4	3.1×10^5
		10.0	1.0	0	3.8×10^4	2.6×10^5
C[f]	*Phytophthora cactorum*			0	0	0
			1.0	3.0×10^2	4.4×10^3	1.1×10^2 (0)[g]
		10.0		1.6×10^2	5.2×10^3	3.2×10^4
		10.0	1.0	2.4×10^2	1.7×10^4	5.8×10^4
	Phytophthora citricola			1.4×10^2	1.2×10^3	0
			1.0	1.0×10^4	1.4×10^3	0
		10.0		8.0×10^2	7.9×10^3	3.3×10^2
		10.0	1.0	1.4×10^2	8.1×10^3	1.4×10^4
	Phytophthora megasperma			4.0×10^2	0	0
			1.0	4.4×10^2	0	0
		10.0		0	2.1×10^3	7.8×10^4
		10.0	1.0	7.9×10^1	3.6×10^3	6.4×10^4

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TABLE 1—*Continued*

Culture protocol	Species	Medium supplement[a]		No. of the following produced/ml[b]:		
		Cholesterol (mg/liter)	Lecithin (g/liter)	Oogonia	Aborted oospores[c]	Viable oospores
	Pythium ultimum			1.2×10^2	4.3×10^3	0
			1.0	2.8×10^3	1.4×10^4	1.2×10^1 (0)[g]
		10.0		2.0×10^1	6.0×10^3	9.4×10^4
		10.0	1.0	1.2×10^1	8.0×10^2	1.8×10^5

[a] The basal growth medium was the defined medium described by Ko (37). Cholesterol was solubilized in tyloxapol-ethanol (1:1) and added to the medium at 2 μl/ml. The lecithin used was synthetic dioleoyl phosphatidylcholine, which was dissolved in a minimal volume of diethyl ether prior to autoclaving.

[b] Oogonia and oospore yields are the means of two replicates. If any single yield varied by more than 15% from the mean, an additional two replicates were performed.

[c] Aborted oospores were those which had formed a persistent oospore wall but had not completed subsequent maturation steps.

[d] For culture protocol A, a 7-mm agar plug from a 10-day-old culture grown on PYG+ was transferred to the center of an agar plate containing the defined growth medium of Ko (37). Liquid cultures for oospore evaluations were inoculated with a 7-mm plug obtained a minimum of 2 cm from the agar inoculum plug on the agar plates.

[e] Culture protocol B was the same as protocol A except that the PYG+ plug was transferred to defined liquid culture medium (37). A minimal quantity of mycelium from the edge of the colony was used to inoculate the culture flasks used for oospore evaluations.

[f] For culture protocol C, the fungi were maintained for seven passages on defined liquid culture medium, and flasks for oospore evaluations were inoculated with a minimal quantity of hyphae from the colony edge.

[g] No viable oospores were formed when dileoyl phosphatidylcholine was further purified on aminopropyl columns prior to being added to the media.

plete maturation requires specific sterol ring and side chain structures and configurations. This differentiation was not made by Ko (37, 38), and we assume that his data referred to the production of viable oospores.

Previous studies with *P. cactorum* have established that the level of sterols necessary for optimum oosporogenesis is ca. 10 mg/liter (15, 16, 49) in variations of a defined medium developed by Elliot (13). The low levels of sterols in the inoculum used in these investigations did not approach 10 mg/liter, and it can be argued that these trace levels had little or no role in inducing the observed levels of oosporogenesis; however, assumptions that all species or all isolates of one species maintained and grown on different media will have comparable sterol requirements (37) are questionable. A synergistic effect of the lecithin added at 1 g/liter with 0.1 mg of cholesterol per liter on viable oospore production was observed for all four pythiaceous fungi (Table 1). For all evaluations this medium or a medium supplemented with the lecithin at 1 g/liter plus 10 mg of cholesterol per liter consistently produced the greatest numbers of viable oospores.

The synergistic effect of cholesterol and dioleoyl phosphatidylcholine on oosporogenesis was investigated in greater detail with *P. ultimum*. Initial investigations revealed that while the 2 μl of aqueous tyloxapol-ethanol per ml used to solubilize cholesterol for the experiments shown in Table 1 had a negligible effect on oosporogenesis when cholesterol at levels greater than ca. 0.1 mg/liter was used in conjunction with the lecithin or when cholesterol at 1.0 mg/liter was used alone (data not shown), as little as 1 μl/ml reduced oosporogenesis at lower levels of sterol supplementation. For a second series of evaluations the cholesterol either was added in diethyl ether and solubilized by the addition of the lecithin in those cases in which both were added or was dispersed in crystalline form without any emulsifier. The absence of a solubilizing agent may have reduced sterol uptake by the mycelium but was not checked as a possible cause of the lower limit set on sterol-induced oosporogenesis in the absence of the lecithin (Table 2).

The primary effect of dioleoyl phosphatidylcholine added alone to defined media was to increase the initiation of oogonia in *P. ultimum* (Table 2). Cholesterol added alone at levels as low as 0.01 mg/liter induced appreciable numbers of viable oospores, with progressive increases in yields as sterol levels were increased up to 10 mg/liter (Table 2). The lecithin added at 1 g/liter acted synergistically with cholesterol present at concentrations of 0.001 mg/liter and higher. Media supplemented with 10 mg of cholesterol and 1 g of the lecithin per liter produced nearly 2.5 times as many viable oospores as did media containing the same level of cholesterol only (Table 2). Lower levels of lecithin supplementation also increased oosporogenesis in media containing 1.0 mg of cholesterol per liter (Table 2). It is not known if solubilization of cholesterol by the lecithin, which is assumed to facilitate its uptake, was responsible in part for the observed synergism.

Effect of lecithin supplementation on the lipid composition of *P. ultimum*. Examination of the PL composition of *P. ultimum* grown on defined media with and without 1 g of dioleoyl phosphatidylcholine per liter revealed that appre-

TABLE 2. Synergistic effect of dioleoyl phosphatidylcholine and cholesterol on oosporogenesis by *P. ultimum*

Medium supplement[a]		No. of the following produced/ml[b]:		
Cholesterol (mg/liter)	Lecithin (g/liter)	Oogonia	Aborted oospores[c]	Viable oospores
		1.3×10^5	3.1×10^3	0
	0.1	1.8×10^5	5.0×10^3	0
	0.5	3.3×10^5	1.1×10^4	8.0×10^3 (0)[d]
	1.0	3.9×10^5	3.3×10^4	1.8×10^4 (0)[d]
0.01		3.4×10^5	9.0×10^3	2.9×10^3
0.1		2.4×10^5	8.7×10^3	2.6×10^4
1.0		1.8×10^3	5.8×10^3	1.1×10^5
10.0		2.1×10^3	7.4×10^3	1.7×10^5
0.001	1.0	1.2×10^5	1.3×10^4	1.4×10^4
0.01	1.0	1.3×10^5	3.2×10^4	3.2×10^4
0.1	1.0	1.2×10^5	1.0×10^4	1.1×10^5
1.0	1.0	1.1×10^4	1.2×10^4	2.8×10^5
10.0	1.0	2.9×10^3	5.0×10^4	4.2×10^5
1.0	0.1	1.8×10^3	1.1×10^3	1.7×10^5
1.0	0.5	3.1×10^3	0.9×10^3	2.7×10^5

[a] Cholesterol was added in a minimal volume of diethyl ether prior to autoclaving. Cultures inoculated with *P. ultimum* were maintained for 12 passages on the defined liquid medium of Ko (37). All other conditions were as described in Table 1, footnote *a*.

[b] See Table 1, footnote *b*.

[c] See Table 1, footnote *c*.

[d] See Table 1, footnote *g*.

TABLE 3. Compositions of major PL classes of *P. ultimum*

Growth medium[a]	PL composition[b]									
	PC		PE		CAEP		PGs		UNK	
	PL/g[c]	% of total PL	PL/g[c]	% of total PL	PL/g[c]	% of total PL	PL/g[c]	% of total PL	PL/g[c]	% of total PL
Basal	0.90	37.9	1.14	47.6	0.29	12.0	0.02	0.9	0.04	1.6
Basal + dioleoyl phosphatidylcholine	0.92	46.7	0.61	31.2	0.20	10.3	0.03	1.6	0.20	10.2
Basal + dioleoyl phosphatidylcholine + V8	0.10	12.5	0.26	31.7	0.42	52.2	0.03	3.6	Tr	Tr

[a] Basal medium was the defined liquid medium of Ko (37). Dioleoyl phosphatidylcholine was added at 1 g/liter. Basal and basal-dioleoyl phosphatidylcholine cultures were inoculated with *P. ultimum* maintained for 15 passages on the defined liquid medium of Ko (37). The basal-dioleoyl phosphatidylcholine-V8 culture was inoculated with 10 7-mm agar disks from a fungal culture maintained on V8 medium.

[b] PE, phosphatidylethanolamine; CAEP, ceramide aminoethylphosphonate; PGs phosphatidylglycerols, including cardiolipin; UNK, unknown PL with an R_f of 2.1 in solvent system 1. Other classes of PL were present in minor quantities.

[c] Fresh weight.

ciable quantities of the phosphatidylcholine were not taken up by the fungus (Table 3). When the two cultures that were inoculated with *P. ultimum* maintained for 15 passages on defined liquid medium (basal and basal plus phosphatidylcholine) were compared, the percentage of total PL made up by the phosphatidylcholine was increased in the lecithin-supplemented cultures; however, the weight of the lecithin present per gram (fresh weight) of mycelium was nearly identical to that in the unsupplemented cultures (Table 3). The enhanced levels of the lecithin were much lower than the 1 g/liter added to the culture media. No viable oospores were visible in either culture prior to lipid extraction.

The most interesting result was the difference in PL composition of a culture of this species grown on basal medium plus dioleoyl phosphatidylcholine when inoculated with agar blocks from a culture maintained on V8 medium. This culture produced abundant viable oospores, in excess of 10^5/ml. Large amounts of sterols are present in V8 juice medium inoculum. In this culture there was an appreciable reduction in the absolute and relative amounts of the phosphatidylcholine (Table 3). This was matched by a twofold increase in the weight of ceramide aminoethylphosphonate and a fivefold increase in the percentage of this PL relative to that found in the culture grown on the same medium in the absence of sterols (Table 3). It should be noted that previous analyses of the PL of *P. ultimum* failed to reveal the presence of this unusual lipid (4). This was probably the result of comigration in thin-layer chromatography of ceramide aminoethylphosphonate with phosphatidylglycerol and lysophosphatidylethanolamine, as previously discussed by Wassef and Hendrix (61).

The major effect of lecithin supplementation was to markedly enhance the levels of oleic acid ($C_{18:1}$) in neutral and polar lipid fractions relative to that found in *P. ultimum* grown on the basal medium (Table 4). This was matched by a marked decrease in palmitic ($C_{16:0}$) and eicosapentaenoic ($C_{20:5}$) acids in the neutral lipid fraction and in myristic ($C_{14:0}$) and palmitic acids in the polar lipid fraction in the lipid-supplemented cultures. There was a significant increase in the percentage of mono- and diunsaturated fatty acids in the lecithin-supplemented cultures.

DISCUSSION

While the role of exogenous PL, especially those with unsaturated fatty acid moieties, in promoting oosporogenesis cannot be denied, the data reported here suggest that recent reports that sterols are not necessary for the induction of mature oospores by some pythiaceous fungi are erroneous. Trace levels of sterols in the inoculum and probably in the growth medium have been shown to be sufficient to induce appreciable levels of oosporogenesis, especially in cultures supplemented with dioleoyl phosphatidylcholine. The studies of Ko (37, 38) simply reiterate the contention of Harnish (18a) studying *P. cactorum*, Hohl (23, 24) working with *Phytophthora* spp., Bahnweg (1) examining *Phytophthora epistomium*, Zaki et al. (62) investigating *Phytophthora cinnamomi*, Kerwin and Washino (28, 31, 32, 34) culturing *L. giganteum*, and Doster and Bostock (12) studying *Phytophthora syringae* that oleic acid and other unsaturated fatty acids and their esters promote growth and/or sexual reproduction by these sterol-auxotrophic fungi. PL in the absence of sterols are not sufficient for the induction and subsequent maturation of oospores.

The role of sterols in mediating morphological and physiological changes in organisms remains an enigma. Sterols

TABLE 4. Fatty acid composition of *P. ultimum*

Growth medium and type of lipid[a]	% Fatty acid composition by wt[b]											
	$C_{14:0}$	$C_{16:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	γ-$C_{18:3}$	α-$C_{18:3}$	$C_{20:0}$	$C_{20:3}$	$C_{20:4}$	$C_{20:5}$
Basal												
Neutral	Tr	22.7	0.6	1.7	18.7	16.4	0.2	0.7	3.4	1.2	11.6	22.9
Polar	17.1	40.2	7.2	1.8	10.2	11.0	0.3			0.2	3.6	8.4
Basal + dioleoyl phosphatidylcholine												
Neutral	7.5	9.6	2.3	0.5	59.2	12.0	Tr	Tr	0.6		3.4	5.0
Polar	3.7	20.3	2.1	11.2	42.4	7.8	Tr	1.1		1.3	8.4	1.7

[a] Basal medium was the defined liquid medium of Ko (37). Each medium (200 ml) was inoculated with *P. ultimum* cultures maintained for 15 passages on the defined liquid medium of Ko (37).

[b] Major isomers of unsaturated fatty acids were of all of the cis configuration. Two isomers of $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, and $C_{20:4}$ were present. The major (>98%) $C_{20:4}$ isomer was 5,8,11,14-eicosatetraenoic acid.

have a well-documented ability to modulate membrane fluidity (2, 3, 10, 52), which affects permeability (25, 40, 43) and protein mobility and enzymic activity (35, 36, 51). In addition to this bulk role of sterols, studies on mycoplasmas (7, 8), the yeast *Saccharomyces cerevisiae* (54, 55), *P. cactorum* (47), and *L. giganteum* (unpublished observations) have implicated a second, more specific sparking effect of these compounds on growth and reproduction. A recent study of *S. cerevisiae* has implicated four separate levels of physiological function for sterols in supporting the growth of this yeast (53). The bulk regulation of membrane fluidity requires ca. 10 to 15 μg of sterols with relatively nonspecific structural features per ml, while the sparking, proposed domain, and critical domain roles require 1 ng to 1 μg of sterols with very specific structures per ml.

The studies reported here and those of Ko (37, 38) suggest that if provided with a suitable source of unsaturated fatty acids, some isolates of *Pythium* and *Phytophthora* spp. require sterols only for the sparking (and perhaps the proposed domain and critical domain) phenomena related to the induction and maturation of oospores. The bulk role is provided for by other molecular species, perhaps similar to the recently described phytophthorols from *P. cactorum* (48).

Changes in membrane PL affect oosporogenesis but not in the sense envisioned by Ko (37), since at least phosphatidylcholine-supplemented cultures of *P. ultimum* do not appear to take up appreciable quantities of the PL headgroup. The radical shift in PL composition in *P. ultimum* cultures grown in identical media but with different inoculum sources could be the result of developmental differences. The observed increase in ceramide aminoethylphosphonate in cultures with trace levels of sterols sufficient to produce large numbers of oospores could reflect a shift away from phosphatidylcholine solely as a result of oospore maturation. It could, however, be the result of different pathways available to the fungus that permit alterations in membrane lipid composition in the presence of trace levels of sterols without disruption of cellular function. There are no developmentally synchronized systems among the pythiaceous fungi which allow a rigorous examination of this question.

Ko (37, 38) documented enhanced oospore yields with either phosphatidylcholine or phosphatidylethanolamine for several fungi. A final argument against these classes of lipid inducing oosporogenesis in the absence of sterols involves the dynamic molecular shapes of these PL. As discussed in detail by Cullis and De Kruijff (6), phosphatidylcholine assumes a fairly cylindrical shape within lipid bilayers. In contrast, phosphatidylethanolamine with unsaturated fatty acid moieties tends to be a cone-shaped molecule with a predilection for nonbilayer configurations. It is difficult to rationalize these disparate molecular shapes modulating physiological processes in the same manner.

It is more likely that a nonspecific phospholipase(s) (phospholipase B) similar to that described for *Phytophthora infestans* (44) hydrolyzed fatty acids from exogenous PL and that the fungus took up primarily or only the fatty acid moieties from the growth medium. This conclusion is supported by our lipid analyses of *P. ultimum*. Enrichment of membrane PL with oleic acid could enhance oosporogenesis in conjunction with sterol-induced processes, as documented for *L. giganteum* (32).

Changes in the lipid macro- and microenvironments of enzymes, receptors, and transporter channels are likely to regulate the complex events underlying the initiation and maturation of oospores (58, 59). The sterol, fatty acid, and PL compositions of membranes are interrelated and can change unpredictably in response to alterations in the chemical and physical environments (9, 26). The initial analyses reported here are further complicated by recent reports of the probable involvement of cyclooxygenase and lipoxygenase products in sexual reproduction by this primitive class of fungi (22, 29). Substrate availability is often the limiting factor in the activity of these enzymes, and the type of PL headgroups, their fatty acid compositions, and the access of phospholipases to appropriate fatty acids will determine in part the morphological development of these fungi. Use of these sterol auxotrophs as model systems will allow new insight into physiological processes underlying lipid-mediated morphogenesis.

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