Sexual Reproduction of *Pythium aphanidermatum*: Stimulation by Phospholipids

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ABSTRACT

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In a chemically defined liquid medium, lecithins obtained from egg yolk (type III-E) were as effective as those obtained from soybean in inducing oospore formation of *Pythium aphanidermatum*. L-α-phosphatidylethanolamine from soybean also induced sexual reproduction, but L-α-phosphatidylinositol from soybean or L-α-phosphatidyl-L-serine from bovine brain did not. Dioleoyl- and dilinoleoyl-L-α-phosphatidylcholine of synthetic lecithins also induced oospore formation in *P. aphanidermatum*. Results suggest that the activity of lecithins is determined by the type and position of fatty acids in the molecular structure. The activity of lecithins was partially replaced by triolein, diolein, dipalmitin, and trilinolein. However, the activity of these glycerides could not be replaced by their corresponding fatty acids.

Additional key words: cephalins, diglycerides, monoglycerides, triglycerides.

In 1964, scientists from several institutes independently reported that sterols are required for sexual reproduction in *Phytophthora* (2,3,5,11) and *Pythium* (4,5). Their observations have since been confirmed and expanded by many researchers from other laboratories and the alleged essentiality of sterols for sexual reproduction in pythiaceous fungi has been cited frequently as factual (1,12,13). Recently, Ko and Ho (10) reported that sterols stimulate but are not essential for sexual reproduction of *Phytophthora cactorum* (Lebert & Cohn) Schroeter and *Phytophthora parasitica* Dastur, because such stimulatory effect can also be produced by lecithins. Subsequently, the phospholipid cephalin has also been shown to be very effective in inducing sexual reproduction of *P. cactorum* (8).

Pythium aphanidermatum (Edson) Fitzp. is one of the species of *Pythium* that has been reported to require sterols for sexual reproduction (6). During the study of the effect of lecithins on other pythiaceous fungi, sterols were also found to be nonessential for sexual reproduction of *P. aphanidermatum*, because such stimulatory effect is also replaceable by lecithins (8). In fact, lecithins were more effective in inducing sexual reproduction in this fungus than were sterols. I report here the effect of various phospholipids on sexual reproduction of *P. aphanidermatum* and the structural requirement for lecithins to stimulate sexual reproduction.

MATERIALS AND METHODS

Media. The basal medium contained 0.1 g of KNO_3, 0.1 g of KH_2PO_4, 0.05 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCO_3$, 10 ml of trace element solution, 0.1 g of asparagine, and 2 g of glucose in 1 L of distilled water (8). The trace element solution contained 100 mg of FeEDTA, 10 mg of $CuSO_4 \cdot 5H_2O$, 10 mg of $MnCl_2 \cdot 4H_2O$, 5 mg of $Na_2MoO_4 \cdot 2H_2O$, 10 mg of $Na_2B_4O_7 \cdot 10H_2O$, 10 mg of $ZnSO_4 \cdot 7H_2O$, and 100 mg of thiamine hydrochloride in 1 L of distilled water. Before the addition of other compounds, the medium was adjusted to pH 8 with 0.5 M KOH, because slightly above neutral pH appeared to be favorable to oospore formation (8).

Phospholipids, glycerides, and fatty acids were purchased from Sigma Chemical Company (St. Louis, MO 63178). These compounds were dissolved in 1 ml of ether individually before being added to the basal medium. Those phospholipids in chloroform or hexane were evaporated to dryness at 24 C in a 50-ml beaker in a fume hood and then redissolved in ether. Before use, 200 ml of ether was washed twice with 400 ml of distilled water by shaking in a 1,000-ml separatory funnel. Those media containing large particles of insoluble compounds were ground in a tissue homogenizer before sterilization. Flasks (250 ml), each containing 10 ml of medium, were covered with aluminum foil and autoclaved for 15 min. Highly purified SeaKem HGT(P) agarose (FMC Corporation, BioProducts, Rockland, ME 04841), which was essentially free of nutrient contaminants (7,10), was used at a concentration of 0.8% (w/v) to solidify the basal medium.

Oospore production. *Pythium aphanidermatum* (isolate 606) obtained from M. Aragaki was maintained on 10% V-8 juice agar (10% V-8 juice, 0.02% $CaCO_3$, 2% Bacto agar). To obtain inocula free from nutrient contaminants, a piece of agar culture (approximately 5 × 5 × 3 mm) was placed on the center of a basal agarose medium in a 90-mm-diameter petri dish. After incubation at 24 C for 4 days, one piece of this culture (approximately 3 × 3 × 3 mm) obtained more than 10 mm from the inoculum was used to seed the media in each flask. After incubation at 24 C in darkness for 10 days, 40 ml of distilled water was added to each flask and the culture was triturated in an Omni mixer at 4,500 rpm for 1 min. The suspension was mixed with 2 drops (approximately 0.1 ml) of 5% (v/v) Tween 80 and the oospore concentration in the suspension was determined by counting the number of spores in 1, 10, or 50 μl with a Pipetman microliter pipette (P-20D or P-200D; West Coast Scientific, Oakland, CA 94618) (9). Two replicates were used for each treatment, and all experiments were repeated at least twice.

RESULTS

Lecithins obtained from egg yolk (type III-E) were as effective as those obtained from soybean in inducing oospore formation of *P. aphanidermatum* (Table 1). Type III-E egg yolk lecithin was more than 10 times more effective than type V-E egg yolk lecithins. Of the three other types of phospholipids tested, only L-α-phosphatidylethanolamine (cephalin) from soybean was effective in inducing oospore formation of *P. aphanidermatum*. Neither L-α-phosphatidylinositol from soybean nor L-α-phosphatidyl-L-serine from bovine brain was effective. Of the four synthetic lecithins tested, *P. aphanidermatum* produced oospores only in media containing dioleoyl- or dilinoleoyl-L-α-phosphatidylcholine. The fungus did not produce oospores in media containing dipalmitoyl- or distearoyl-L-α-phosphatidylcholine.

The basal liquid medium was supplemented with 100 μg/ml of choline chloride, 100 μg/ml of K_2HOP_4 and 1,000 μg/ml of individual glyceride to study the effect of glycerides on sexual

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reproduction of *P. aphanidermatum*. *P. aphanidermatum* formed a moderate number of oospores in media containing triolein, diolein, dipalmitin, or trilinolein (Table 2). No oospores were formed when other glycerides, including monoolein, tripalmitin, monopalmitin, dilinolein, monolinolein, tristearin, distearin, and monostearin were tested. The fungus also failed to form oospores when the glycerides in the above media were replaced by 100 μg/ml of glycerol and 1,000 μg/ml of oleic acid, palmitic acid, or stearic acid, or 100 μg/ml of linoleic acid. Linoleic acid completely inhibited growth of *P. aphanidermatum* at the concentration of 1,000 μg/ml.

DISCUSSION

Although the natural lecithins used in this study were only 99% pure, the possibility that a sterol present as an impurity in the lecithins is responsible for the stimulatory effect rather than the lecithins per se has been shown to be unlikely in a previous report concerning the stimulatory effect of lecithins on oospore formation by *Phytophthora cactorum* (8). It was found that even if the 1% impurity in the soybean lecithins is entirely sterols, such sterol impurity would account for less than 2% of the oospores produced by *P. cactorum* in the presence of lecithins. Moreover, the stimulatory spectrum of lecithins was different from that of sterols. Lecithins were stimulatory to sexual reproduction of *Phytophthora capsici* and *P. aphanidermatum* but not *Pythium vexans*, whereas sterols were stimulatory to *P. aphanidermatum* and *P. vexans* but not *P. capsici*. This suggests that the mode of action of lecithins in inducing sexual reproduction in fungi is different from that of sterols (8).

The fact that in addition to natural lecithins, cephalins, two types of synthetic lecithins, and four types of glycerides were all effective in inducing oospore formation by *P. aphanidermatum* supports the previous report that sterols are stimulatory to but not essential for sexual reproduction of *P. aphanidermatum* (8). According to criteria of essentiality, an essential substance cannot be replaced by any other substance (10).

Since L-α-phosphatidylethanolamine was as effective as the lecithins in inducing sexual reproduction of *P. aphanidermatum*, the choline component of the lecithins was assumed not to be associated with their activity. The activity is apparently determined by the type and position of fatty acids in the molecular structure of lecithin because of the four different kinds of synthetic lecithins tested, only those containing oleic acid or linoleic acid were effective. Lecithins containing palmitic acid and stearic acid were ineffective. Because dioleoyl-L-α-phosphatidylcholine and dilinoleoyl-L-α-phosphatidylcholine were only about one-third as active as soybean lecithins, the type and position of the fatty acids in the molecular structure of the lecithins that are most effective for sexual reproduction of *P. aphanidermatum* remain to be determined.

Present results also showed that the activity of lecithins can be partially replaced by certain glycerides. However, the activity of glycerides cannot be replaced by their corresponding fatty acids. Only certain glycerides of oleic acid, palmitic acid, and linoleic acid were effective in inducing sexual reproduction of *P. aphanidermatum* and none of the glycerides of stearic acid tested was effective. This suggests that the ability of glycerides to induce sexual reproduction is also determined by the type and position of fatty acids in the molecular structure.

Sexual reproduction of *P. cactorum* also was stimulated by lecithins and cephalins from soybean but was not affected by L-α-phosphatidylinositol from soybean or L-α-phosphatidyl-L-serine from bovine brain (8). However, unlike *P. aphanidermatum*, *P. cactorum* did not form oospores in basal liquid medium containing dilinoleoyl-L-α-phosphatidylcholine. Triolein and trilinolein also failed to induce oospore formation by *P. cactorum*.

TABLE 1. Oospore formation by *Pythium aphanidermatum* in a basal liquid medium supplemented with 1,000 μg/ml of various phospholipids

Phospholipid	Product no.[a]	Purity (%)	Oospores/ml medium
Different sources of L-α-lecithins			
Soybean, type III-S	P6263	99	44,400
Egg yolk, type III-E	P5388	99	58,800
Egg yolk, type V-E	P5763	99	4,500
Other types of phospholipids			
L-α-phosphatidylethanolamine, soybean	P5274	98	48,100
L-α-phosphatidylinositol, soybean	P5766	98	0
L-α-phosphatidyl-L-serine, bovine brain	P7769	98	0
Synthetic lecithins			
Dioleoyl-L-α-phosphatidylcholine	P1013	99	12,500
Dipalmitoyl-L-α-phosphatidylcholine	P6769	99	0
Dilinoleoyl-L-α-phosphatidylcholine	P7649	99	12,500
Distearoyl-L-α-phosphatidylcholine	P6517	99	0
None			0

[a]Sigma Chemical Company, St. Louis, MO 63178.

TABLE 2. Oospore formation by *Pythium aphanidermatum* in a basal liquid medium supplemented with 100 μg/ml of choline chloride, 100 μg/ml of K_2HPO_4 and 1,000 μg/ml of various glycerides

Glyceride	Product no.[a]	Purity (%)	Oospores/ml medium
Triolein	T7140	99	2,200
Diolein	D3380	99	4,817
Monoolein	M7765	99	0
Tripalmitin	T5888	99	0
Dipalmitin	D2636	99	10,875
Monopalmitin	M1640	99	0
Trilinolein	T9517	99	3,942
Dilinolein	D9508	95	0
Monolinolein	M7640	99	0
Tristearin	T5016	99	0
Distearin	D9019	99	0
Monostearin	M2015	99	0
None			0

[a]Sigma Chemical Company, St. Louis, MO 63178.

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