Stimulation of Sexual Reproduction of *Phytophthora cactorum* by Phospholipids

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In a chemically defined liquid medium, soybean lecithins (99% pure) were much more effective than β -sitosterol and cholesterol in stimulating oospore formation by isolate Pct N of *Phytophthora cactorum* at 20 and 24 °C. Assuming that the 1% impurity is all sterols, such sterol impurity would account for less than 2% of the oospores produced in the presence of lecithins. Lecithins were also stimulatory to sexual reproduction of *Phytophthora capsici* and *Pythium aphanidermatum* but not *Pythium vexans*, whereas sterols were stimulatory to sexual reproduction of *Pythium aphanidermatum* and *Pythium vexans* but not *Phytophthora capsici*. Lecithins from soybean were more effective in inducing sexual reproduction by isolate 121F of *Phytophthora cactorum* than were those from egg yolk. L- α -Phosphatidylethanolamine from soybean was as effective as lecithins in inducing sexual reproduction, whereas L- α -phosphatidylinositol from soybean and L- α -phosphatidyl-L-serine from bovine brain were ineffective. Of four synthetic lecithins tested, only dioleoyl-L- α -phosphatidylcholine was active. The triglycerides and fatty acids tested were inactive. The results suggest that the activity of lecithins is determined by the type and position of fatty acids in the molecular structures.

INTRODUCTION

Since 1964 when scientists from four different institutes (Elliott *et al.*, 1964; Harnish *et al.*, 1964; Hendrix, 1964; Leal *et al.*, 1964) independently reported observations that addition of sterols to basal agar media induced sexual reproduction of both self-inducing and cross-inducing species of *Phytophthora*, the alleged essentiality of sterols for sexual reproduction of *Phytophthora* has been cited frequently as factual (Smith & Berry, 1974; Barnett, 1976; Webster, 1980). Their reports have spurred research activity in this field with *Phytophthora cactorum* as the most commonly used test organism (Hendrix, 1970; Elliott, 1983). Recently, Ko & Ho (1983) reported that sterols stimulate, but are not essential for, sexual reproduction of *P. cactorum* and *Phytophthora parasitica* because such a stimulatory effect can also be produced by lecithins. I report here new evidence for the role of lecithins in sexual reproduction of *P. cactorum* and the structural requirement for lecithins to stimulate sexual reproduction.

METHODS

Organisms. Isolates 121F and Pct N of *Phytophthora cactorum* were supplied by D. L. McIntosh (Canadian Department of Agriculture, Summerland, BC, Canada) and W. D. Nes (USDA, Berkeley, Calif., USA) respectively. The A¹ mating type of *Phytophthora capsici* is a sexual variant originating from aged culture of the A² mating type (isolate P287) supplied by M. Aragaki (Ko, 1981) who also supplied isolates 606 of *Pythium aphanidermatum* and 530 of *Pythium vexans*.

Media. The basal medium contained the following: KNO₃, 0.1 g; KH₂PO₄, 0.1 g; MgSO₄·7H₂O, 0.05 g; CaCO₃, 0.1 g; trace elements, 10 ml; asparagine, 0.1 g; glucose, 2 g; and distilled water, 1 l. The following trace elements were supplied per litre: FeEDTA, 100 mg; CuSO₄·4H₂O, 10 mg; NaMoO₄·2H₂O, 5 mg; Na₂B₄O₇·10H₂O, 10 mg; ZnSO₄·7H₂O, 10 mg; thiamine hydrochloride, 100 mg. The basal medium was adjusted to pH 8 with 0.5 M-KOH before the addition of other compounds. Cholesterol and β -sitosterol were

obtained from Calbiochem and ICN, respectively. Phospholipids, triglycerides and fatty acids were purchased from Sigma. These compounds were dissolved in ether individually (10 mg ml^{-1}) 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 dissolved in ether. Before use, 200 ml ether was washed twice with 400 ml distilled water by shaking in a 1000 ml separation funnel. Those media containing large particles of insoluble compounds were ground in a tissue homogenizer before sterilization. Flasks (250 ml) each containing 10 ml medium were covered with aluminium foil and autoclaved for 15 min. Highly purified Seakem agarose (HGT-P Agarose; Marine Colloids) which was essentially free of nutrient contaminants (Ho & Ko, 1980; Ko & Ho, 1983) was used to solidify the basal medium at a concentration of 0.8% (w/v).

Oospore production. A piece of agar culture (approx. $5 \times 5 \times 3 \text{ mm}$) from each fungus growing on 10% V-8 agar [10% (v/v) V-8 vegetable juice, 0.02% (w/v) CaCO_3 and 2% (w/v) Bacto-agar] was placed on the centre of a basal agarose medium in a Petri plate. After incubation at 24°C for 4 d, pieces of culture (approx. $3 \times 3 \times 3 \text{ mm}$) obtained more than 10 mm from the inoculum were used to inoculate the media. For *P. capsici*, each flask of medium was inoculated with two pieces each of A¹ and A² mating type cultures. For the other species, each flask of medium was inoculated with one piece of culture. After incubation at 24°C in darkness for 10 d, 40 ml distilled water was added to each flask and the culture was triturated in an Omni mixer at 4500 r.p.m. for 1 min. The suspension was mixed with 2 drops (approx. $50 \mu\text{l}$ each) 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 $100 \mu\text{l}$ with a Pipetman microlitre pipette (P-20D or P200D; West Coast Scientific, Oakland, Calif., USA; Ko *et al.*, 1973). Two replicates were used for each treatment and all experiments were repeated at least twice.

RESULTS AND DISCUSSION

Lecithins at $1000 \mu\text{g ml}^{-1}$ induced formation of 15667 oospores of *P. cactorum* isolate Pct N per ml of medium at 20°C , whereas β -sitosterol and cholesterol at $10 \mu\text{g ml}^{-1}$ induced formation of only 206 and 275 oospores per ml of medium, respectively (Table 1). At 24°C , the amounts of oospores induced by lecithins, β -sitosterol and cholesterol were 4667, 25 and 90 per ml of medium, respectively.

Since β -sitosterol is the common sterol in soybean (Windholz, 1976) and is one of the most active sterols (with an optimum concentration of about $10 \mu\text{g ml}^{-1}$) in inducing sexual reproduction of *P. cactorum* (Elliott, 1972; Elliott *et al.*, 1966), these results suggest that lecithins were much more effective than sterols in stimulating oospore formation by isolate Pct N of *P. cactorum*. Although the lecithins used 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* is unlikely. 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.

The stimulatory spectrum of lecithins was different from that of sterols (Table 2). Lecithins were stimulatory to sexual reproduction of *P. capsici* and *Pythium aphanidermatum* but not *Pythium vexans*, whereas sterols were stimulatory to *Pythium aphanidermatum* and *Pythium 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. Sterols have also been considered to be essential for sexual reproduction of *Pythium* species including *Pythium aphanidermatum* (Hendrix, 1965, 1970; Elliott, 1983). This result shows that the stimulatory effect of sterols on sexual reproduction of *Pythium aphanidermatum* can also be produced by lecithins. Therefore, as with *Phytophthora*, sterols are stimulatory to, but not essential for, sexual reproduction of *Pythium aphanidermatum*. Sexual reproduction of *Pythium vexans* was induced by sterols but not lecithins. However, whether sterols are essential for sexual reproduction in this fungus is not known because other substances have not been tested.

Since isolate 121F of *P. cactorum* produced many more oospores in the presence of lecithins than isolate Pct N (Ko & Ho, 1983), the former was used in the determination of the structural requirement for lecithins to stimulate sexual reproduction. Lecithins obtained from soybean were more effective than those obtained from egg yolk in inducing oospore formation by isolate 121F of *P. cactorum* (Table 3). When the concentration of soybean lecithins was decreased from 1000 to 100 and $10 \mu\text{g ml}^{-1}$, the number of oospores produced per ml of medium decreased from 59000 to 663 and 0, respectively. This may explain why Elliot *et al.* (1966) did not find lecithins

Table 1. Oospore formation by isolate Pct N of *Phytophthora cactorum* in a defined liquid medium containing 1000 µg lecithins ml⁻¹ or 10 µg sterols ml⁻¹ at 20 and 24 °C

Stimulatory substance	No. of oospores per ml of medium at:	
	20 °C	24 °C
L-α-Lecithins (Sigma P6263, 99%)	15667	4667
β-Sitosterol (ICN 102886, 81.7%)	206	25
Cholesterol (Calbiochem 228111, 99%)	275	90
None	0	0

Table 2. Oospore formation by species of *Phytophthora* and *Pythium* in a defined liquid medium containing 1000 µg lecithins ml⁻¹ or 10 µg sterols ml⁻¹ at 24 °C

Stimulatory substance*	No. of oospores per ml of medium		
	<i>Phytophthora capsici</i> †	<i>Pythium aphanidermatum</i>	<i>Pythium texans</i>
L-α-Lecithins	221	67417	0
β-Sitosterol	0	13050	10360
Cholesterol	0	12760	14500
None	0	0	0

* The same as Table 1.

† Pairing between A¹ and A² mating types.Table 3. Oospore formation by isolate 121F of *Phytophthora cactorum* in a defined liquid medium containing 1000 µg ml⁻¹ of various phospholipids at 24 °C

Phospholipid	No. of oospores per ml of medium
Different sources of L-α-lecithins	
Soybean, type III-S (Sigma P6263, 99%)	59000
Egg yolk, type III-E (Sigma P5388, 99%)	3200
Egg yolk, type V-EA (Sigma P5763, 99%)	12900
Other types of phospholipids	
L-α-Phosphatidylethanolamine (Sigma P5274, soybean, 98%)	75000
L-α-Phosphatidylinositol (Sigma P5766, soybean, 98%)	0
L-α-Phosphatidyl-L-serine (Sigma P7769, bovine brain, 98%)	0
Synthetic lecithins	
Di-oleoyl-L-α-phosphatidylcholine	6900
Dipalmitoyl-L-α-phosphatidylcholine	0
Dilinoleoyl-L-α-phosphatidylcholine	0
Distearoyl-L-α-phosphatidylcholine	0
None	0

stimulatory to sexual reproduction of *P. cactorum* as they used 10 µg lecithins ml⁻¹. Of the three other types of phospholipids tested, only L-α-phosphatidylethanolamine (cephalin) from soybean was effective in inducing oospore formation of *P. cactorum* (Table 3). Neither L-α-phosphatidylinositol from soybean nor L-α-phosphatidyl-L-serine from bovine brain was effective. Their ineffectiveness was not due to the presence of inositol or serine because addition of 200 µg myo-inositol ml⁻¹ or 100 µg L-serine ml⁻¹ to basal medium plus 1000 µg ml⁻¹ of soybean lecithins did not reduce the number of oospores produced by *P. cactorum*. About 67000 and 80000 oospores per ml of medium were produced in the presence of myo-inositol and L-serine, respectively. Of the four synthetic lecithins tested only di-oleoyl-L-α-phosphatidylcholine was effective in inducing sexual reproduction of *P. cactorum* (Table 3). Dipalmitoyl-, dilinoleoyl- and

distearoyl-L- α -phosphatidylcholines were ineffective. *P. cactorum* did not form oospores in basal medium plus 100 μg K_2HPO_4 ml^{-1} , 100 μg choline chloride ml^{-1} and 1000 μg ml^{-1} of individual triglycerides including triolein, tripalmitin, trilinolein, trilinolenin and tristearin. The fungus also failed to form oospores when triglycerides in the above media were replaced by 100 μg glycerol ml^{-1} and 1000 μg ml^{-1} of their corresponding fatty acids.

Since L- α -phosphatidylethanolamine was as effective as lecithins in inducing sexual reproduction of *P. cactorum*, the choline component was not associated with the activity of lecithin. The activity is probably determined by the type and position of fatty acids in the molecular structure of lecithin because lecithins from egg yolk were not as active as lecithins from soybean. Moreover, of the four different kinds of synthetic lecithins tested, only the lecithin containing oleic acid was effective. Those lecithins containing other types of fatty acids were ineffective. Since dioleoyl-L- α -phosphatidylcholine was about one-tenth as active as soybean lecithins, the type and position of the fatty acids in the molecular structures of the lecithins which are most effective for sexual reproduction of *P. cactorum* remain to be determined. Results from this study also showed that the activity of lecithins probably cannot be replaced by triglycerides or fatty acids.

Lecithins have been reported by Hohl (1975) to be stimulatory to vegetative growth of several species of *Phytophthora* including *P. cactorum*. Synthetic lecithins were also ineffective or less effective than natural lecithins in promoting growth of *P. cactorum*. However, contrary to sexual reproduction, the growth promoting activity of lecithins in *P. cactorum* was totally replaced by linoleic acid.

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